Cellular/Molecular

A Retinal-Specific Regulator of G-Protein Signaling Interacts with $G\alpha_o$ and Accelerates an Expressed Metabotropic Glutamate Receptor 6 Cascade

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G_o is the most abundant G-protein in the brain, but its regulators are essentially unknown. In retina, $G\alpha_{o1}$ is obligatory in mediating the metabotropic glutamate receptor 6 (mGluR6)-initiated ON response. To identify the interactors of G_o, we conducted a yeast two-hybrid screen with constituitively active $G\alpha_o$ as a bait. The screen frequently identified a regulator of G-protein signaling (RGS), Ret-RGS1, the interaction of which we confirmed by coimmunoprecipitation with $G\alpha_o$ in transfected cells and in retina. Ret-RGS1 localized to the dendritic tips of ON bipolar neurons, along with mGluR6 and $G\alpha_{o1}$. When Ret-RGS1 was coexpressed in *Xenopus* oocytes with mGluR6, $G\alpha_{o1}$, and a GIRK (G-protein-gated inwardly rectifying K^+) channel, it accelerated the deactivation of the channel response to glutamate in a concentration-dependent manner. Because light onset suppresses glutamate release from photoreceptors onto the ON bipolar dendrites, Ret-RGS1 should accelerate the rising phase of the light response of the ON bipolar cell. This would tend to match its kinetics to that of the OFF bipolar that arises directly from ligand-gated channels.

Key words: **ON bipolar neuron; retina; $G\alpha_o$; Ret-RGS1; GTPase-activating protein; GIRK channel**

Introduction

A photoreceptor signals dimming and brightening by increasing and decreasing its release of glutamate. This fluctuating output is then transmitted to two classes of second-order neuron, one that depolarizes to glutamate (OFF bipolar cell) and another that hyperpolarizes (ON bipolar cell). In mammals the ON bipolar cells account for 75% of all bipolar cells and are responsible for both night and day vision (Sterling et al., 1988; Cohen and Sterling, 1990; Martin and Grünert, 1992; Strettoi and Masland, 1995). Thus it is important to understand the molecular basis for response of ON cells to light and glutamate. It is now established that the ON response is mediated by turning off a G-protein cascade that is active in darkness when glutamate binds to the metabotropic glutamate receptor 6 (mGluR6) (Nomura et al., 1994; Vardi and Morigiwa, 1997; Vardi et al., 2000). The heterotrimeric G-protein has been identified as G_{o1} (Nawy, 1999; Dhingra et al., 2000, 2002), which during activation somehow closes a nonspecific cation channel (Nawy and Jahr, 1990; Shiells and Falk, 1990, 1992; de la Villa et al., 1995; Euler et al., 1996). Key questions are how G_o affects the channel and how the cascade is regulated.

G_o is the most abundant G-protein in the brain (Sternweis and Robishaw, 1984; Huff et al., 1985; Li et al., 2000). It is involved in many signal transduction cascades, ranging from regulating several ion channels (voltage-gated Ca^{2+}, K^+, and possibly a cGMP-dependent channel) to regulating multiple aspects of behaviors in *Caenorhabditis elegans* (Kleuss et al., 1991; Mendel et al., 1995; Berghard and Buck, 1996; Kojima et al., 1997; Valenzuela et al., 1997; Gomez and Nasi, 2000; Greif et al., 2000). Except for certain Ca^{2+} and K^+ channels, however, where the $\beta\gamma$ subunits of G_o directly bind the channel (Dolphin, 1998; Leaney and Tinker, 2000), the mechanisms of the transduction of G_o are virtually unknown. The purpose of this study was to elucidate the mGluR6 cascade by finding interactors of $G\alpha_{o1}$ in retina.

Using active $G\alpha_{o1}$ as bait in a yeast-two hybrid system, we identified a regulator of G-protein signaling (RGS), Ret-RGS1, which is a retina-specific splice variant of RGS20 (Faurobert and Hurley, 1997; Barker et al., 2001). After confirming the interaction by coimmunoprecipitation, we localized Ret-RGS1 to ON bipolar dendrites, in which mGluR6 and $G\alpha_{o1}$ are coexpressed. Expressing Ret-RGS1 with mGluR6 and $G\alpha_{o1}$ in *Xenopus* oocytes, we found that Ret-RGS1 accelerates the termination of the cascade. Ret-RGS1 is also expressed by photoreceptor terminals, where it may serve a different function.

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Materials and Methods

Yeast two-hybrid screen

Yeast two-hybrid screen was performed using cDNA encoding constitutively active $G\alpha_{o1}$ Q205L in pAS2–1 vector (Clontech, Cambridge, UK). The bovine retinal cDNA library (in pACT2–1) was generously provided by Dr. Ching-Hwa Sung (Cornell Medical School, Ithaca, NY). The presence of $G\alpha_{o1}$ protein in yeast transformed with $G\alpha_{o1}$ plasmid was checked by Western blots. Approximately 2×10^6 yeast clones cotransformed with $G\alpha_{o1}$ and library plasmid were screened by Matchmaker GAL4 Two-Hybrid system (BD Biosciences, Palo Alto, CA). The colonies growing on SD/-His/-Leu/-Trp medium (BD Biosciences) were further assayed for LacZ. To eliminate false positives, in the initial screen, positive clones were subjected to cycloheximide counterselection to obtain colonies that had lost the bait plasmid and retained the library plasmid. These clones were then remated with clones carrying $G\alpha_{o1}$ (in pACT2–1), pACT2–1 vector alone, or pLAMC (negative control). The positive clones from remating were then grown, and DNA was transformed into *Escherichia coli* for sequencing. The insert was sequenced using primers in the pACT2–1 vector; these included rod and cone phosphodiesterase γ (PDEγ) and Ret-RGS1.

RT-PCR

Retinal RNA was prepared using High Pure RNA Isolation kit (Roche, Mannheim, Germany). The reverse transcription was performed on 1 μg of total RNA with oligo dT primers by using Moloney murine leukemia virus reverse transcriptase (BD Biosciences). The PCR reaction was performed using degenerate primers for mouse cone and rod PDEγ (forward: cagttcaagagcaagccncccaag; reverse: actgngcnagctc(g/a)tgcagctc). Thirty-five cycles (94°C for 1 min, 54°C for 1 min, and 72°C for 2 min) were performed on a programmable thermocycler (PerkinElmer Life Sciences, Boston, MA).

Colony hybridization

The yeast clones to be screened were grown on a master plate in a grid pattern and lifted onto nylon membrane followed by denaturation and neutralization by following the Matchmaker two-hybrid system supplier's protocol (BD Biosciences). Digoxigenin-labeled cone and rod PDEγ probes were made using PCR Dig Probe Synthesis kit (Roche). Hybridization was performed at 42°C in Dig Easy Hyb buffer with gentle agitation overnight followed by two 15 min washes (moderate stringency) in 0.5× SSC, 0.1% SDS at 68°C. Immunological detection was performed with anti-Dig antibody (Roche).

Primary antibodies

The antibody against Ret-RGS1 was raised against the bovine N1 peptide (see Fig. 1*A*) and has been characterized (Faurobert et al., 1999; this study). Anti-$G\alpha_o$ was a mouse monoclonal antibody (mAb) raised against the bovine protein (mAb 3073; Chemicon, Temecula, CA). Anti-$G\alpha_{o1}$ was a rabbit polyclonal raised against the specific peptide EYPGSNTYED (gift from D. Manning, University of Pennsylvania, Philadelphia, PA). The specificity of the monoclonal and polyclonal anti-$G\alpha_o$ has been established (Vardi et al., 1993; Dhingra et al., 2002). Anti-protein kinase C (PKC)α (clone MC5; Amersham Biosciences, Little Chalfont, UK), anti-calbindin (clone CB-955; Sigma, St. Louis, MO), and anti-synaptophysin (mAb 5258; Chemicon) were mouse monoclonal antibodies. Polyclonal anti-Na/K ATPase was a gift from K. Geering (Institute of Pharmacology and Toxicology, Lausanne, Switzerland).

Immunocytochemistry

Bovine eyes were purchased from a local slaughterhouse and placed on ice until further treatment. An eye was hemisected along the ora serata, everted, and immersed in 2% or 4% buffered paraformaldehyde (0.1 M phosphate buffer, pH, 7.4) for 1 hr. After a rinse, the eye was cryoprotected in 30% sucrose in phosphate buffer overnight at 4°C; the retina was detached, embedded in a 2:1 mixture of 20% buffered sucrose and tissue freezing medium, and cryosectioned radially at 10–15 μm thickness. Sections were preincubated in 0.1 M phosphate buffer containing 10% normal goat serum, 5% sucrose, and 0.3% Triton X-100, incubated in primary antibody for 48–72 hr at 4°C, rinsed, incubated in a secondary antibody conjugated to a fluorescent marker (Jackson ImmunoResearch, West Grove PA), rinsed, and mounted in Vectashield (Vector Laboratories, Burlingame, CA). Digital images were acquired using a confocal microscope (Leica, Exton, PA). For immunoelectron microscopy, 100 μm radial Vibratome sections were immunostained as above except that Triton X-100 (0.1%) was added only to the preincubation medium (omitting Triton X-100 gave no staining). Sections were incubated with secondary antibody conjugated to HRP (anti-rabbit Fab fragments conjugated to HRP), developed using diaminobenzidine and hydrogen per-

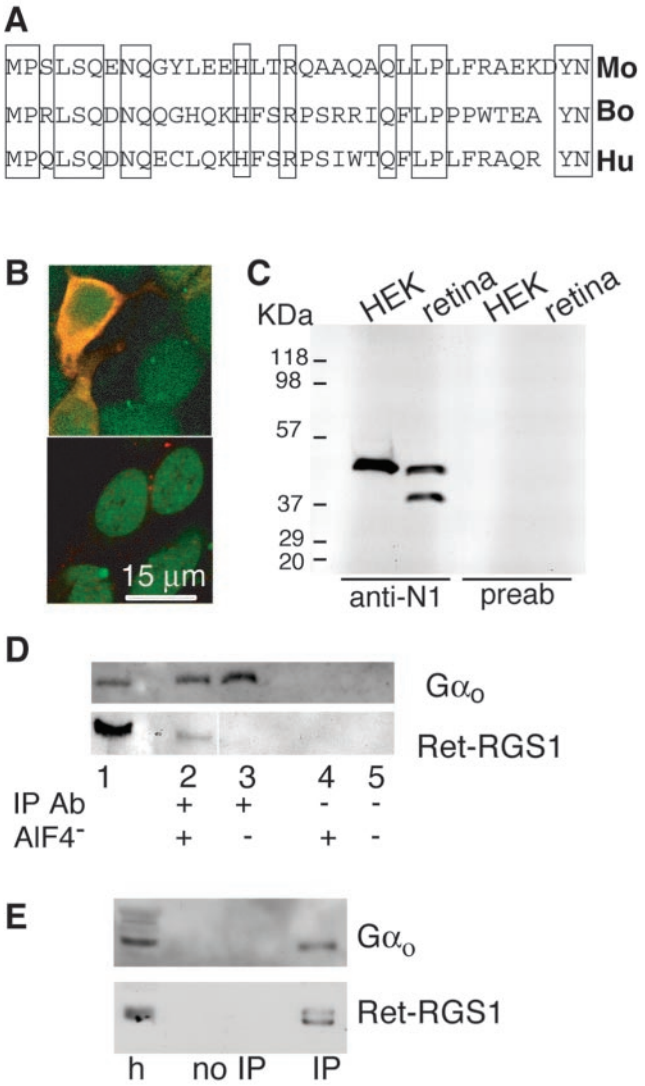

Figure 1. $G\alpha_o$ interacts with Ret-RGS1. *A*, Alignment of amino acid residues of mouse (Mo), bovine (Bo), and human (Hu) Ret-RGS1 protein N termini (corresponding to the N1 bovine peptide used to generate the antibody against Ret-RGS1). *B*, HEK 293 cells transfected with bovine Ret-RGS1 expression plasmid (top) and mouse Ret-RGS1 EST clone (bottom), immunostained with N1 antibody (red) and counterstained with the nuclear dye SYTO 13 (green). This antibody recognized the bovine protein (top) but not the mouse protein (bottom). *C*, A Western blot stained with N1 antibody shows two bands of 45 and 40 kDa in bovine retina and a 45 kDa band in HEK cells transfected with Ret-RGS1 expression plasmid. These bands were eliminated by preabsorbing the antibody with the N1 peptide (preab). *D*, Coimmunoprecipitation of $G\alpha_{o1}$ and Ret-RGS in HEK cells transfected with $G\alpha_{o1}$ and Ret-RGS1 expression vectors. $G\alpha_o$ was immunoprecipitated in the presence or absence of AlF_4^- (as indicated). The immunoprecipitates were subjected to Western blotting with $G\alpha_o$ (top) or Ret-RGS1 (bottom) antibodies in parallel. 1, Homogenate; 2 and 3, immunoprecipitation (IP) with anti-$G\alpha_o$; 4 and 5, mock IP without any antibody. In bottom blot, an empty lane next to lane 2 was cut out for alignment. For both *D* and *E*, 0.3% of total sample was applied for the homogenate lane, and 5% was applied for the rest of the lanes. *E*, Coimmunoprecipitation of $G\alpha_o$ and Ret-RGS1 from bovine retina. IP was done with anti-$G\alpha_o$ in the presence of AlF_4^-. h, Homogenate.

oxide, and intensified using the gold-substitution silver intensification method (Johnson and Vardi, 1998). The sections were then treated with osmium tetroxide (2%; 60 min), stained with 1% uranyl acetate in 70% ethanol, dehydrated in graded ethanol series (70–100%), cleared in propylene oxide, and embedded in Epon 812. Ultrathin sections were mounted on Formvar-coated slot grids and stained with uranyl acetate.

Cell culture and transfection

Human embryonic kidney (HEK) 293 cells were cultured in minimal essential medium supplemented with Penstrep (Invitrogen, Carlsbad, CA) and 10% heat-inactivated fetal bovine serum at 37°C in a 5% CO_2 incubator. Cells were transiently transfected with $G\alpha_{o1}$ (in pDP; provided by D. Manning, University of Pennsylvania) and Ret-RGS1 (in pRC) expression plasmids using Fugene transfection reagent (Invitrogen). Cells were harvested 48 hr later.

Coimmunoprecipitation

Transfected cells or frozen bovine retinas were collected in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 5 mM MgCl2, 100 μM GDP, 30 mM PMSF) with or without AlF_4^- (30 μM $AlCl_3$ and 10 mM NaF). The cells were homogenized at low speed and centrifuged at 8000 × *g* in an Eppendorf centrifuge for 5 min. The supernatant was precleared by adding 20 μl of Protein G agarose beads (Invitrogen), centrifuging, and collecting the supernatant. The precleared supernatant was incubated with mouse anti-$G\alpha_o$ and Protein G agarose beads on a rotator at 4°C for ~12 hr. The beads with protein complexes were then pulled down by centrifuging (10,000 × *g*), washed thoroughly in lysis buffer, resuspended in Lammelli buffer, boiled, and spin filtered. The proteins were run on 10% SDS-PAGE gel and transferred to a nitrocellulose membrane using semiwet transfer apparatus (Bio-Rad, Hercules, CA). Blots were then incubated sequentially in the following: 10% nonfat dry milk in PBS containing 0.1% Tween 20 (PBST) for 1 hr at 4°C, primary antibodies (against $G\alpha_{o1}$ or Ret-RGS1) in PBST at 4°C overnight, PBST, secondary antibodies (anti-rabbit Fab fragments linked to HRP; Protos, Burlingame, CA), and detected by SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology, Rockford, IL).

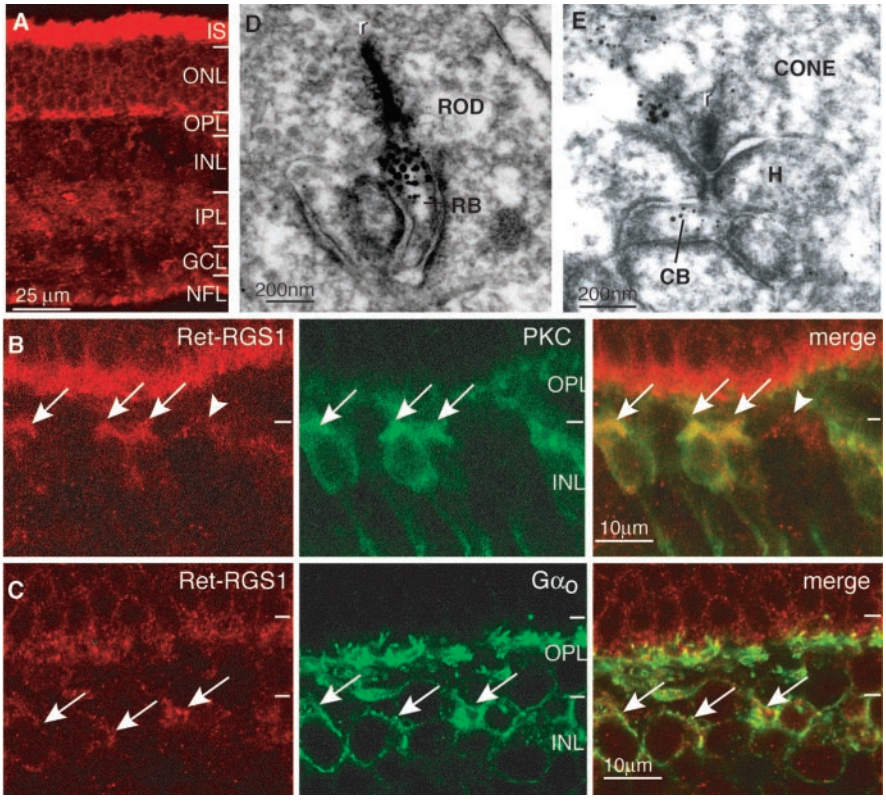

Figure 2. Ret-RGS1 is localized to ON bipolar somas and dendrites. *A*, Radial section of bovine retina stained for Ret-RGS1. Staining is present in all retinal layers, most strongly in inner segments and then OPL. Staining in IPL and ganglion cell somas was somewhat variable between tissues. IS, Inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; NFL, nerve fiber layer. (Abbreviations apply to all Figures.) *B*, Double staining for Ret-RGS1 (red) and PKC (green). Rod bipolar cells marked with anti-PKC (arrows) were also stained for Ret-RGS1. Certain PKC-negative somas were Ret-RGS1 positive (arrowhead). *C*, Double staining for Ret-RGS1 (red) and $G\alpha_o$ (green). All ON bipolar cells marked by $G\alpha_o$ express Ret-RGS1 (arrows). *D*, Electron micrograph of a rod synaptic complex. The invaginating rod bipolar dendritic tip (RB) is stained for Ret-RGS1. r, Ribbon. *E*, Electron micrograph of a cone synaptic complex. The central element of the postsynaptic triad (CB) was stained for Ret-RGS1. H, Horizontal cell process.

Fractionation of bovine retinas and purification of synaptic vesicles and plasma membrane on sucrose gradients

Retinas for this purpose were obtained from François Le Madec (of the slaughterhouse of Puget-Théniers, France). The following procedures were modified from the standard protocol for purification of synaptic vesicles from mammalian brain (Huttner et al., 1983).

Preparation of crude synaptosomes. Freshly isolated bovine retinas were disrupted in ice-cold homogenization buffer (320 mM sucrose, 1 mM $MgCl_2$, 4 mM HEPES–NaOH buffer, pH 7.3) supplemented with protease inhibitors (Complete; Roche Diagnostics) and 50 μg/ml DNase I (Roche Diagnostics) by shear force with a Polytron (Kinematica, Littau, Switzerland). The homogenate was centrifuged (10 min; 800 × *g*), and the supernatant (S1) was collected and centrifuged again (15 min; 9200 × *g*) to yield a pellet P2 and a supernatant S2. An aliquot of S2 was centrifuged again (30 min; 400,000 × *g*) to yield a "cytosol" supernatant. The pellet (P2) was washed and centrifuged (15 min; 10,200 × *g*). The subsequent pellet was frozen and thawed in homogenization buffer to yield the synaptosomal fraction.

Fractionation of synaptosomes. The crude synaptosomal fraction was further homogenized, dissolved in Tris-HCl (10 mM, pH 6.8), and diluted in Tris-HCl (pH 6.8), KI, and KCl to a final concentration of 100 mM, 0.6 M, and 0.1 M, respectively. The lysate was further centrifuged (20 min; 25,000 × *g*) to yield a pellet containing lysed synaptosomal membranes and a supernatant containing synaptic vesicles.

Purification of synaptic vesicles. The supernatant was centrifuged (2 hr; 65,000 × *g*), the resulting supernatant, referred as synaptosomal cytosol, was removed, and the pellet containing the crude synaptic vesicles was washed in 10 mM HEPES, pH 7.4, and centrifuged (15 min; 400,000 × *g*). The pellet was homogenized in 40 mM sucrose and passed through a 23 gauge needle. The suspension was then layered on top of a linear continuous sucrose gradient, which was centrifuged in a SW41 Beckman rotor (5 hr; 65,000 × *g*). Fractions were collected from the bottom of the tube, concentrated by speed-vac, and precipitated with methanol/chloroform.

Purification of synaptic plasma membranes. Synaptic plasma membranes were prepared as described in Herms et al. (1999). In brief, the lysed synaptosomal membranes were resuspended in 1.2 M sucrose and loaded on discontinuous sucrose gradient containing 5 ml of 0.8 M sucrose and 1 ml of 0.3 M sucrose. After centrifugation at 65,000 × *g* for 2.5 hr, the synaptic plasma membranes band between 0.8 and 1.2 M sucrose was collected, diluted in ice-cold water, and centrifuged (30 min; 48,000 × *g*).

Tests of solubilization of Ret-RGS1 from retina membranes

A bovine retina was homogenized in hypotonic buffer containing 5 mM Tris-HCl, pH 7.5, and protease inhibitors (Complete; Roche Diagnostics). The homogenate was centrifuged (15 min; 800 × *g*) at 4°C. The supernatant was dispensed equally in six microcentrifuge tubes and cen-

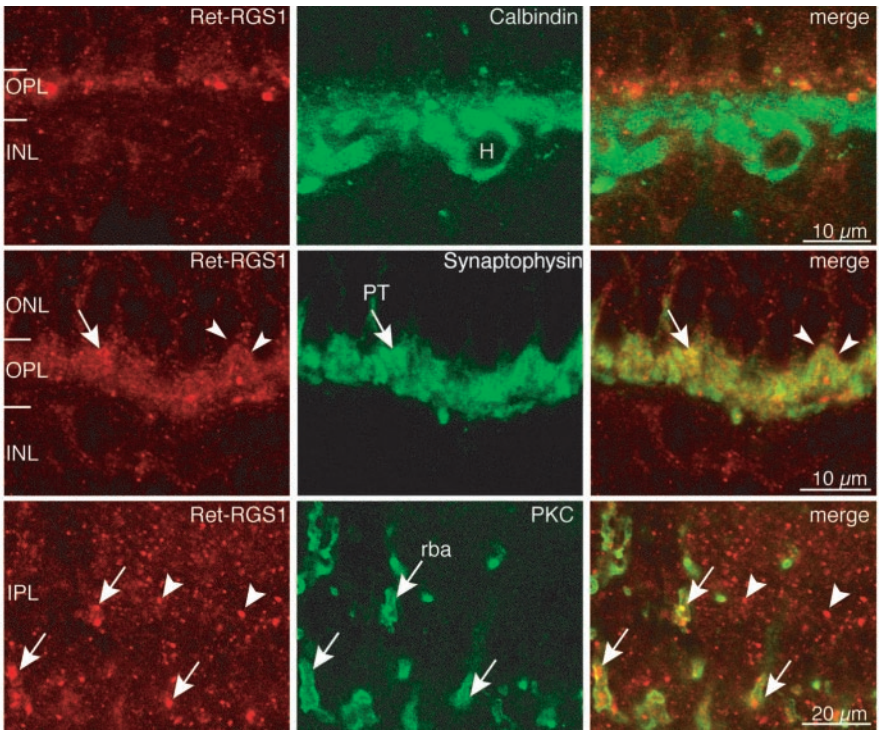

Figure 3. Ret-RGS1 is expressed in presynaptic terminals. Top row, Immunostaining for Ret-RGS1 (red) and horizontal cell marker, calbindin (green). Horizontal cell processes are unstained for Ret-RGS1. Middle row, Immunostaining for Ret-RGS1 (red) and synaptophysin (green). Ret-RGS1 and synaptophysin colocalize in presynatic photoreceptor terminals (PT) (arrows). Note that Ret-RGS1 staining forms clusters, which often outline the synaptophysin-positive terminals (arrowheads). Bottom row, Immunostaining for Ret-RGS1 (red) and PKC (green). Staining for Ret-RGS1 forms large puncta throughout the IPL (arrowheads). Rod bipolar axon terminals (rba) (identified by PKC) are also stained (arrows).

trifuged at 25,000 × *g* for 20 min. The resulting membrane pellets were resuspended at 2 mg/ml in six different buffers: hypotonic (5 mM Tris-HCl, pH 7.5), hypertonic (Tris-HCl, pH 7.5, 1 M NH_4Cl), sodium carbonate (100 mM Na_2CO_3, pH 11.6), urea (5 mM Tris-HCl, pH 7.5, 4 M urea), detergent (5 mM Tris-HCl, pH 7.5, 50 mM n-octyl glucoside), and hydroxylamine (1 M NH_2OH, pH 7.5). The membranes were homogenized by extrusion through a 23 gauge needle, incubated on ice for 20 min, and centrifuged for 15 min in a TLA100.1 rotor at 400,000 × *g*. For hydroxylamine washing, the suspension was incubated for 18 hr at room temperature. Equivalent amounts of supernatant and pellet were analyzed by immunoblot on a 12% polyacrylamide gel.

cDNA constructs and RNA

The coding sequences of all cDNA used for oocyte injection were prepared or obtained as described previously (Sharon et al., 1997; Vorobiov et al., 2000). All cDNAs were inserted into high-expression oocyte vectors containing 5′ and 3′ untranslated sequences of *Xenopus* β-globin: pGEM-HJ [Muscarinic 2 receptor (m2R), G-protein-gated inwardly rectifying K^+ channel (GIRK)1, GIRK2, $G\alpha_{o1}$, Ret-RGS1], or pBluescript SK(−) (mGluR6). Plasmids were linearized with *Not*I, and mRNA was transcribed *in vitro* using a cap structure at the 5′ end site (Yakubovich et al., 2000). The plasmid containing the Ret-RGS1 coding sequence was subcloned from pRC/CMV into pGEM-HJ using PCR and inserting an *Eco*RI sequence at the 5′ end and a *Hind*III sequence at the 3′ end. Pertussis toxin (PTX)-insensitive $G\alpha_{o1}$ mutants (C351I and C351V) were prepared with the Quick Change Site Directed Mutagenesis kit (Stratagene, La Jolla, CA).

Xenopus *oocyte recordings*

Xenopus laevis oocytes were maintained and injected as described (Dascal and Lotan, 1992; Dascal, 2000). mRNA was injected 3 d before experiments, and oocytes were maintained at 20°C in ND96 (96 mM NaCl, 2 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 5 mM HEPES, pH 7.5) supplemented with 2.5 mM Na-pyruvate and 50 μg/ml gentamicin. Pertussis toxin (200 pg) was injected 12–16 hr before the experiment. An oocyte was placed in a chamber (~50 μl), impaled with two sharp electrodes with tips that were filled with 1% agarose plus 3 M KCl (resistance ~0.3 MΩ), and immediately perfused with ND96 at 0.2–0.4 ml/sec. The holding potential was set to −80 mV. Basal and evoked currents were measured in a high potassium solution containing (in mM): 24.5 KCl, 73.5 NaCl, 1 $CaCl_2$, 1 $MgCl_2$, 5 mM HEPES, pH 7.4. An oocyte was tested only once to remove variability caused by desensitization. Data were acquired with Axotape and pCLAMP software (Axon Instruments, Foster City, CA); statistical analysis (one way ANOVA followed by two-tailed Student's *t* test) was done with Microsoft Excel. Data are presented as either the mean or normalized mean ± SE. To normalize data pooled from two experiments or more, each data point within a batch was divided by control values of this batch, and then normalized values were combined and analyzed as above.

Results

Identifying interactors of constitutively active $G\alpha_{o1}$

To identify interactors of $G\alpha_{o1}$, we screened a bovine retinal cDNA library in a GAL4-based yeast two-hybrid system with a constitutively active α subunit of G_{o1} ($G\alpha_{o1}^*$). An initial screen of ~5×10^4 clones gave 36 positives, in which >90% encoded the γ subunit of photoreceptor PDE. A full screen of ~2×10^6 clones, after hybridizing out the photoreceptor PDEγ-encoding clones, gave ~1000 positive clones, of which we sequenced ~300. These fell into several categories: "potential effector": rod PDEγ (87 hits), cone PDEγ (41); "regulator of G-protein signaling": Ret-RGS1 (37), RAP1-GTPase-activating protein (GAP) (2), RGS10 (3), RGS16 (18), Gα-interacting protein (GAIP) (2); "guanine nucleotide exchange factor"-like proteins: Synembryn (3), activator of G-protein signaling 3 (8); and "guanine nucleotide dissociation inhibitors": Purkinje cell protein-2 (28). Several of these interactors were identified previously by yeast two-hybrid using various nonretinal libraries (Jordan et al., 1999; Luo and Denker, 1999; Natochin et al., 2001, Tall et al., 2003); however, PDEγ, Ret-RGS1, RGS10, and RGS16 are identified here as new potential interactors.

The finding of PDEγ might have been important because the effector of G_o in ON cells was initially suggested to be a phosphodiesterase (Nawy and Jahr, 1990, 1991; Shiells and Falk, 1990); however, immunostainings with three antibodies against PDEγ were negative in bipolar cells, and our efforts to identify a non-photoreceptor isoform using degenerate primers amplified only photoreceptor isoforms. Presumably $G\alpha_o$ interacts with PDEγ because of the homology of G_o to transducin ($G\alpha_t$), which in phototransduction binds PDEγ. The next important interactor was Ret-RGS1, which was cloned previously from cow and human (Faurobert and Hurley, 1997; Barker et al., 2001). The rest of this report concerns Ret-RGS1.

$G\alpha_o$ interacts with Ret-RGS1 in HEK cells and retina

To test the interaction between G_o and ret-RGS1 and localize ret-RGS1, we used a polyclonal antibody raised against the first

35 amino acids of bovine Ret-RGS1 (N1 peptide) (Faurobert et al., 1999), but first we had to establish its specificity. Alignment of the bovine against the corresponding mouse (sequence obtained from Sheryll Barker, University of Texas, Dallas, TX; unpublished data) and human Ret-RGS1 shows a poor match (Fig. 1*A*). We then transfected HEK 293 cells with an expression plasmid coding for bovine Ret-RGS1 or mouse Ret-RGS1 EST clone (IMAGE clone 5363855). The antibody recognized only the bovine form (Fig. 1*B*). Western blots of bovine retinal protein gave a prominent band at ~45 kDa, in agreement with its predicted molecular weight (Fig. 1*C*, left). It also gave a band at 40 kDa. Western blots of transfected HEK cells gave a predominant band of 45 kDa and on longer exposure a weak 40 kDa band. Both 45 and 40 kDa bands were preabsorbed with 50-fold excess of antigenic peptide (Fig. 1*C*, right), suggesting that the 40 kDa band is a proteolytic degradation product, or possibly another splice variant of this gene. Thus, the N1 antibody is specific for bovine Ret-RGS1 but not for mouse. Consequently, we restricted all experiments with this antibody to bovine.

To test whether Ret-RGS1 and $G\alpha_{o1}$ are true interactors, we cotransfected HEK 293 cells with expression plasmids coding for $G\alpha_{o1}$ and bovine Ret-RGS1 and immunoprecipitated $G\alpha_o$ with monoclonal anti-$G\alpha_o$. Under these conditions, a barely detectable amount of Ret-RGS1 coimmunoprecipitated with $G\alpha_{o1}$, but when AlF_4^- [$G\alpha$-GDP-AlF_4^- complex stabilizes the interaction between $G\alpha$ and RGS proteins (Berman et al., 1996)] was added to the protein mix, the signal was stronger (Fig. 1*D*, bottom, lane 2 vs 3). The level of immunoprecipitated $G\alpha_o$ was similar under both conditions (top, lane 2 vs 3). In the absence of immunoprecipitating antibody, the two proteins were undetectable (lanes 4 and 5) (four experiments). This experiment shows that $G\alpha_{o1}$ and Ret-RGS1 can interact in mammalian cells. Next, we examined whether $G\alpha_o$ and Ret-RGS1 interact in the native tissue. Immunoprecipitating $G\alpha_o$ from bovine retina in the presence of AlF_4^- also precipitated Ret-RGS1 (Fig. 1*E*) ($n = 2$). When the immunoprecipitating antibody was omitted, Ret-RGS1 was undetectable. Finally, in the converse experiment, immunoprecipitating Ret-RGS1 with its antibody also precipitated $G\alpha_o$ ($n = 1$). This experiment shows that $G\alpha_{o1}$ and Ret-RGS1 interact in the native tissue.

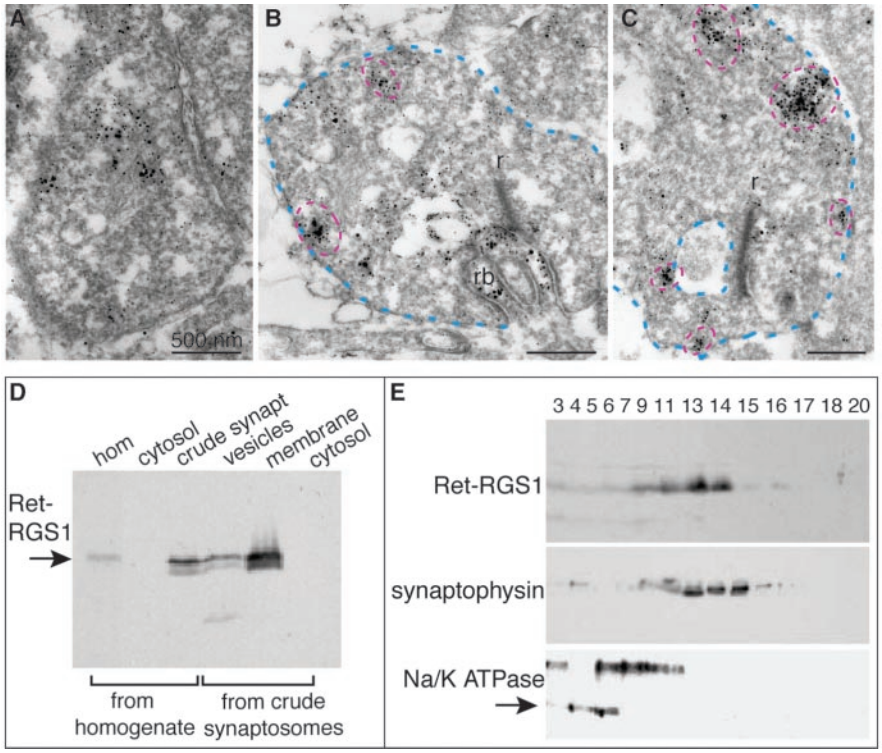

Figure 4. Ret-RGS1 associates with plasma membrane and synaptic vesicles in presynaptic terminals. *A–C*, Electron micrographs of rod terminals. *A*, Diffused stain appears throughout the terminal; *B*, *C*, clusters of gold particles (denoted within the dashed pink ellipses) appear ~100–200 nm from plasma membrane (outlined in blue). *D*, Western analysis of subfractions from bovine retina with Ret-RGS1 antibody. Fifty micrograms of total proteins of each fraction were loaded on a 12% acrylamide gel. Ret-RGS1 is present in plasma membrane fraction and synaptic vesicles. Hom, Homogenate; crude synapt, crude synaptosomes. *E*, Fractions of synaptic vesicles on a sucrose gradient analyzed by Western blotting. Ret-RGS1 is co-present with synaptophysin in medium weight fractions; Na/K ATPase α-subunit (96 kDa; bottom band), a marker of a plasma membrane, settles in the heavier fractions. The 120 kDa (top band) is an aggregate of the α-subunit, which is commonly seen after sample boiling. As for Ret-RGS1, its migration was not perturbed by sample boiling.

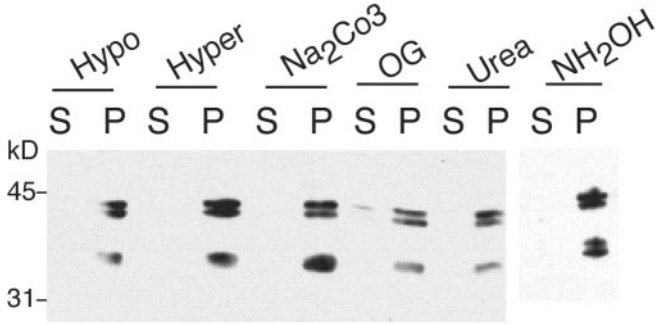

Figure 5. Ret-RGS1 is strongly bound to the plasma membrane. Retinal membranes from 25,000 × *g* pellets were washed with different treatments: hypotonic (hypo), hypertonic (hyper), alkaline (Na_2CO_3), octyl-glucoside (OG), urea, and NH_2OH. For most treatments, Ret-RGS was recovered only in the pellet fraction (P); a small amount of Ret-RGS1 in supernatant (S) was recovered only after octyl-glucoside treatment. This suggests that Ret-RGS1 is tightly associated with the plasma membrane.

Expression pattern of Ret-RGS1

Immunostaining for Ret-RGS was present in many retinal cells: photoreceptor inner segments and cell bodies, outer synaptic layer, bipolar and amacrine somas, and inner synaptic layer (Fig. 2*A*). Omitting the primary antibody or preabsorbing it with the N1 peptide eliminated staining. This general pattern agreed with *in situ* hybridization (Faurobert and Hurley, 1997). This suggests that the stain was specific.

ON bipolar cells express Ret-RGS1 in their dendritic tips

If Ret-RGS1 interacts with $G\alpha_{o1}$ during the light response, it must be present in ON cells. To test this, we costained bovine retina for Ret-RGS1 and PKC, a known marker for rod bipolar cells, which comprise more than half of all ON cells. In the bipolar soma layer, all PKC-positive somas were positive for Ret-RGS1; however, certain somas were negative for PKC but positive for Ret-RGS1

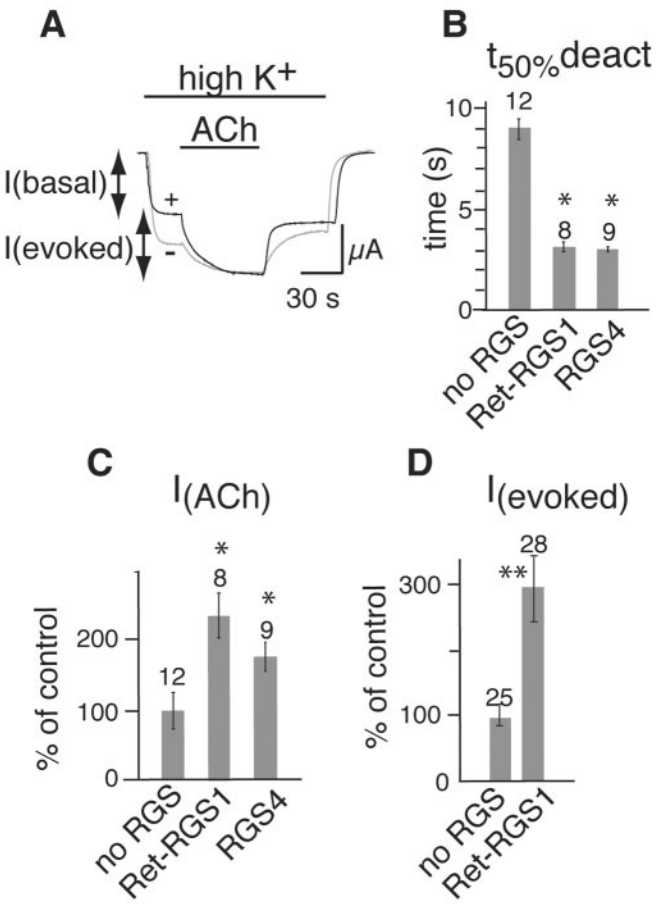

Figure 6. Ret-RGS1 modulates acetylcholine-evoked response. *A*, Experimental protocol (current responses to 10 μM acetylcholine). Bars above the records denote application of high K^+ and acetylcholine solutions. Before and after application of high K^+, oocytes were perfused with ND96 solution. Basal and evoked currents are defined on the left. Gray and black records correspond to records without (−) or with (+) 1 ng Ret-RGS RNA; current scale: 4 μA for − and 8 μA for +. Oocytes were injected with RNA for (in nanograms) m2R (0.24), $G\alpha_{o1}$ (1), and GIRK1/2 channels (0.8 each). For all RNAs in this and the following figures, the amounts are given in nanograms. *B*, *C*, Same experiment as in *A* showing mean and SEM for one experiment. Ret-RGS1 (1) and RGS4 (1) reduced deactivation half-time ($t_{50\%}$ deact) (*B*) and increased ACh-evoked current (*C*) significantly. Above each bar is the number of oocytes. For all figures, a single asterisk denotes a significant difference from control ($p < 0.05$) and double asterisks denote a highly significant difference ($p < 0.001$). *D*, Ret-RGS1 increased the evoked current when interacting with the wild-type $G\alpha_o$ [normalized average of 3 experiments; oocytes were injected with RNA (in nanograms) for m2R (0.2) or mGluR6 (1), $G\alpha_o$ (0 or 1), GIRK1/2 channels (0.8 each), and Ret-RGS1 (0 or 1)].

(Fig. 2*B*). To identify these cells, we costained sections for $G\alpha_o$ (expressed in all ON cells) (Vardi, 1998) and Ret-RGS1. In the inner nuclear layer, all somas positive for $G\alpha_o$ also stained for Ret-RGS1 (Fig. 2*C*).

A further requirement for Ret-RGS1 to regulate $G\alpha_o$ is that it be present in the dendritic tips of the ON cell, where mGluR6 detects glutamate and activates $G\alpha_o$. Electron micrographs of tissue stained for Ret-RGS1 confirmed its presence in dendritic tips that invaginate the rod and the cone synaptic terminals. These invaginating dendrites are known to arise from ON cells (Fig. 2*D*,*E*). No stain was found in the tips of OFF dendrites located at the base of cone terminals. We conclude that all ON cells (but no OFF cells) coexpress Ret-RGS1 in exactly the site where it could regulate the mGluR6 cascade.

Ret RGS1 is present in photoreceptor and rod bipolar terminals and localized to vesicles and plasma membrane

Certain structures in the outer plexiform layer stained strongly for Ret-RGS1 but not for $G\alpha_o$. Horizontal cells, identified by the anti-calbindin antibody (Chun and Wassle, 1993), stained weakly for Ret-RGS1 in the soma but not in the dendrites (Fig. 3, top row); however, photoreceptor terminals, identified by synaptophysin (Brandstätter et al., 1996), were strongly positive for Ret-RGS1 (Fig. 3, middle). In the inner plexiform layer, staining for Ret-RGS1 was punctate throughout the layer. Examination of sections costained for PKC and Ret-RGS1 proved that some of these were located within rod bipolar terminals (Fig. 3, bottom).

Although Ret-RGS1 and synaptophysin were both present in photoreceptor terminals, their fine expression patterns differed. Aggregates of Ret-RGS1 stain appeared to outline the synaptophysin stain (Fig. 3, middle row, arrowheads). Electron microscopy confirmed that Ret-RGS1 was present in photoreceptor terminals (Fig. 4*A*) and showed that it often clustered at the plasma membrane (Fig. 4*B*,*C*). Similar results were obtained by fractionation experiments. After centrifugation of bovine retina, Ret-RGS1 was absent from the cytosol fraction (corresponding to 400,000 × *g* soluble proteins) but present in the crude synaptosomal fraction. Subsequent fractionation of these synaptosomes in the presence of potassium iodide, an actin depolymerizing agent, revealed that most of the Ret-RGS1 protein was associated with the plasma membrane and the rest with synaptic vesicles (Fig. 4*D*). When synaptic vesicles were fractionated on a sucrose gradient, Ret-RGS1 colocalized with synaptophysin (Fig. 4*E*). Plasma membrane, marked by Na/K ATPase, settled lower in the gradient. This shows that the fractions that were strongly positive for synaptophysin and Ret-RGS1 were not contaminated by plasma membrane.

Ret-RGS1 is strongly bound to membranes

The demonstration by immunostaining that Ret-RGS1 associates with membrane seemed consistent with its amino acid sequence. This contains a putative transmembrane domain plus a cysteine string that might target the protein to membrane. To further investigate this association, we treated a crude fraction of bovine retinal membrane with buffers of various ionic strengths (as described for RGS9) (Cowan et al., 1998). These mild treatments (hypotonic and hypertonic washes), that would remove weakly bound proteins, did not solubilize Ret-RGS1, nor did more stringent treatments (sodium carbonate buffer at pH 11.6 denaturation with urea). Treatments that release proteins linked to membranes by palmitoylation (hydroxylamine) also did not release Ret-RGS1. Only when the membranes themselves were solubilized (octyl-glucoside) was a small amount of Ret-RGS1 recovered in the supernatant (Fig. 5). The low solubility of Ret-RGS1 in detergent could be explained either by its precipitation during solubilization and its subsequent partitioning in the 400,000 × *g* pellet or by its association with the actin cytoskeleton; however, the colocalization of Ret-RGS1 with plasma membranes and synaptic vesicles in conditions that disrupt the actin cytoskeleton (Fig. 4*D*) strongly supports the hypothesis that Ret-RGS1 genuinely associates with membranes. One cannot exclude, however, an additional binding to cytoskeletal elements.

Ret-RGS1 accelerates termination of the mGluR6 cascade

So far we had established that Ret-RGS1 localizes to dendritic tips of ON bipolar cells at the same site as mGluR6 and its obligatory transducer, G_{o1}. Next we asked whether Ret-RGS1 can modulate this cascade and how. To test this, we coexpressed (in *Xenopus*

oocytes) Ret-RGS1, mGluR6, and a $G\alpha_{o1}$ that was engineered to be insensitive to PTX. The endogenous $G\alpha_{i/o}$ was then blocked with PTX. To read out the kinetics of G_{o1}, we also expressed the inwardly rectifying potassium channel, GIRK. This channel opens during binding $G\beta\gamma$, which occurs when an agonist-bound receptor activates the trimeric G-protein to release $G\beta\gamma$. The kinetics of activation and especially deactivation of the agonist-induced response depend crucially on GTPase activity of the $G\alpha$ that contributes $G\beta\gamma$, and this activity is typically regulated by RGS.

The general protocol was as follows. An oocyte was voltage clamped to −80 mV, and then its perfusion solution (ND96) was switched from low K^+ (2 mM) to high K^+ (24 mM). This caused an inward current representing basal GIRK activity. When the basal current reached plateau, agonists were added to the perfusate. This evoked a large inward current that turned off when the agonist was removed (Fig. 6*A*).

Because RGS proteins had been tested in this system using a muscarinic receptor (m2R) and the endogenous $G_{i/o}$ (Doupnik et al., 1997; Saitoh et al., 1997), we tested this first. When m2R was activated by 10 μM acetylcholine followed by atropine wash (10 μM), both RGS4 and Ret-RGS1 reduced the deactivation half-time from ~10 to 3 sec. Interestingly, both RGS proteins at the expressed level (1 ng RNA) increased the evoked current, rather than reducing it (Fig. 6*C*). This also occurred when mGluR6 was substituted for m2R and activated by 1 mM glutamate (Fig. 6*D*).

Next we blocked the endogenous $G\alpha_{i/o}$ with PTX and tested Ret-RGS1 against the PTX-insensitive exogenous $G\alpha_{o1}$ (Fig. 7*A*). Larger amounts of mGluR6 RNA gave larger glutamate-evoked currents (Fig. 7*B*). With 0.2–1 ng of mGluR6 RNA and 1 ng of Ret-RGS1 RNA, the evoked responses were diminished drastically, and deactivation half-time could not be measured. With 2 ng of mGluR6 RNA and 1 ng of Ret-RGS1 RNA, the responses were reduced to ~70% ($n = 8$), and the deactivation half-time fell from 18.6 ± 1.3 to 10.2 ± 2.4 sec ($n = 10$). Activation time was unaffected (data not shown). Then, using a constant amount of mGluR6 RNA (2 ng), we tested different amounts of Ret-RGS1 (0.2–2 ng mRNA). Ret-RGS1 reduced deactivation half-time in a concentration-dependent manner up to approximately twofold (Fig. 7*C*,*D*). This effect was obtained with two different PTX-insensitive mutants of $G\alpha_{o1}$ ($G\alpha_{o1}$ C351V and $G\alpha_{o1}$ C351I; five experiments). Ret-RGS1 also mildly reduced activation time and basal current (Fig. 7*E*). With respect to evoked current, lower quantities of Ret-RGS1 (0.5–1 ng RNA) did not change the evoked current, but higher quantities (2 ng) decreased it. When the ratio of evoked to basal currents (a measure of signal-to-noise) was calculated, certain amounts of Ret-RGS1 (0.5 ng) actually increased this ratio. We also compared several RGS proteins (at 1 ng RNA) coexpressed with mGluR6 and $G\alpha_{o1}$ and found effects on evoked current and deactivation half-time as follows: Ret-RGS1>RGS7>RGS4 (Fig. 8). It is possible that a constant amount of RNA did not result in a constant amount or optimal folding of each RGS protein. Nevertheless, this experiment suggests that, at least under the conditions tested, Ret-RGS1, which is normally associated with this cascade, was the most effective regulator.

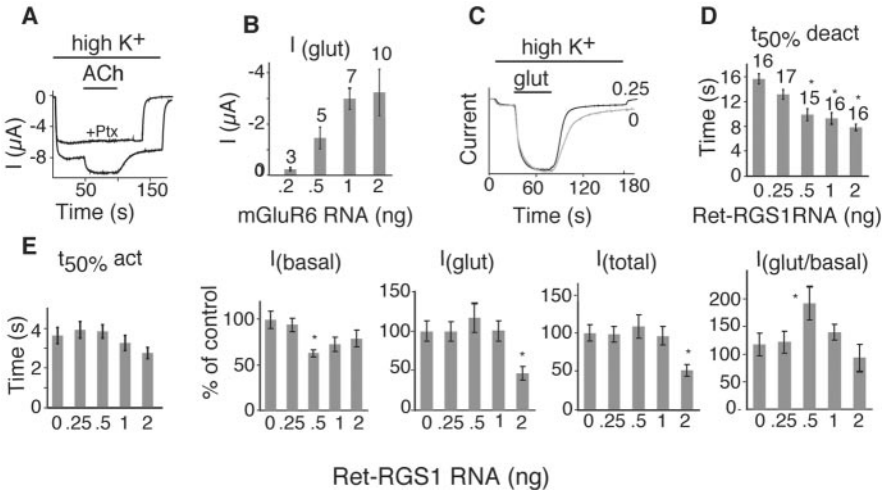

Figure 7. Ret-RGS1 accelerates deactivation of mGluR6 response in a concentration-dependent manner. Pertussis toxin (200 ng) was injected ~16 hr before the recordings. *A*, PTX completely diminished responses carried by endogenous $G\alpha_{i/o}$ protein. *B*, Current evoked by 1 mM glutamate versus quantity of injected mGluR6 RNA. Responses first grow linearly and then saturate ~1 ng mRNA. Oocytes were injected with RNAs (in nanograms) for mGluR6 (as indicated), pertussis toxin-insensitive $G\alpha_{o1}$ C351V (1) and GIRK1/2 channels (0.8 each). *C*, Ret-RGS1 (0.25 ng) accelerated deactivation. Currents were normalized to the same amplitude to show effect of time course. *D*, *E*, Responses to glutamate with increasing amounts of Ret-RGS1 (data pooled from 2 experiments). The parameters quantified are denoted above the bar graphs. Act, activation. Cells were injected with mRNAs for mGluR6 (2), pertussis toxin-insensitive $G\alpha_{o1}$ C351V (1), GIRK1/2 channels (0.8 each), and the indicated amount of Ret-RGS1. For activation and deactivation half-time, data were simply averaged. For currents, data were normalized to responses at 0 Ret-RGS1 as explained in Materials and Methods. Numbers of oocytes denoted in *D* apply also to *E*.

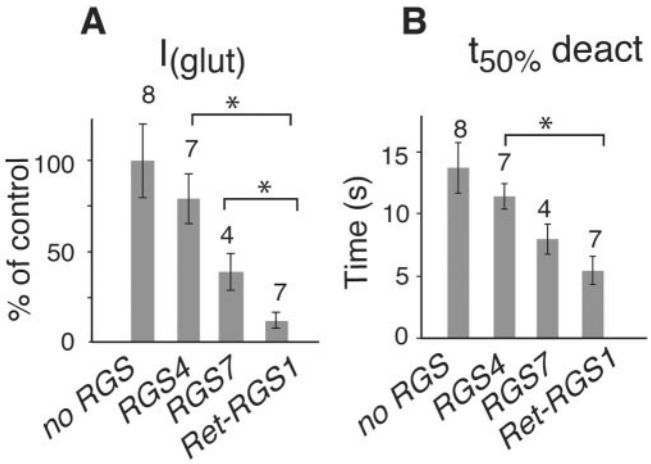

Figure 8. Ret-RGS1 is more potent than RGS4 and RGS7. *A*, *B*, Current responses (*A*) and deactivation half-time (*B*) in the absence or presence of RGS4, RGS7, or Ret-RGS1. Oocytes were injected with RNAs (in nanograms) for mGluR6 (1), pertussis toxin-insensitive $G\alpha_{o1}$ C351V (1), GIRK1/2 channels (0.8 each), and Ret-RGS1 (1), RGS4 (1), or RGS7 (1).

Discussion

We show here that Ret-RGS1 is expressed by ON bipolar cells at the tips of their dendrites, where the mGluR6→$G\alpha_{o1}$ cascade closes cation channels in the dark. We are confident that immunostaining for Ret-RGS1 is specific because the antibody against Ret-RGS1 (1) stains HEK cells transfected with bovine Ret-RGS1 expression plasmid, (2) gives a prominent band at the expected molecular weight in Western blots, (3) staining in Western blots and retinal tissue is completely blocked by the immunogen, and

(4) staining perfectly fits the pattern observed with *in situ* hybridization (Faurobert and Hurley, 1997).

We show further that Ret-RGS1 interacts with $G\alpha_{o1}$ in yeast, cotransfected HEK cells, and native retina. The two-hybrid assay that repeatedly identified Ret-RGS1 never identified the RGS9 of the photoreceptor, although ~70% of retinal messages originate from photoreceptors. This suggests a preference of $G\alpha_{o1}$ for the particular RGS with which it colocalizes *in situ*. Finally, we show in an expression system that Ret-RGS1 can serve as a GAP for $G\alpha_{o1}$ to accelerate response termination. In the expressed muscarinic system, Ret-RGS1 was as effective as RGS4 in accelerating the deactivation of the m2R response; however, in the mGluR6 system, Ret-RGS1 was more potent than RGS4. Furthermore, although Ret-RGS1 can be a GAP for transducin ($G\alpha_t$; the phototransduction G-protein), the rate constant is ~50-fold slower than with the natural GAP of $G\alpha_t$, RGS9 (Grunwald et al., 1986; Lerea et al., 1986,1989; Faurobert and Hurley, 1997; He et al., 1998; Chen et al., 2000; Lyubarsky et al., 2001). Thus, the naturally colocalized pairs, $G\alpha_t$-RGS9 and $G\alpha_{o1}$-Ret-RGS1, interact more strongly than their alternative combinations.

Because an RGS curtails the activity of a G-protein (Berman et al., 1996; Ross and Wilkie, 2000; Chidiac and Roy, 2003), it should reduce the amplitude of agonist-evoked current. Indeed, we observed this in many experiments, but in several, the evoked current was either constant or increased with certain levels of Ret-RGS1. This apparent paradox has been reported for several other RGS proteins. One possibility is that Ret-RGS also reduced the basal current (see below). This might allow more channels to be activated by the agonist and partially explain the nonreduction of evoked current; however, this could not be the only reason, because the total current increased when the evoked current increased, indicating another effect. Note that the effect on the evoked current depends on the precise stoichiometry of receptor, $G\alpha$, and RGS. For example, under the conditions of Figure 8, where injected mGluR6 RNA was 1 ng, Ret-RGS1 (1 ng) dramatically reduced the evoked current, whereas under the conditions of Figure 7, *D* and *E*, where injected mGluR6 RNA was 2 ng, evoked current was larger and Ret-RGS1 hardly affected the evoked current. The increase in evoked current is thought to arise from a direct interaction of the RGS protein with the receptor or the channel (Doupnik et al., 1997; Saitoh et al., 1997; Jeong and Ikeda, 2001; Keren-Raifman et al., 2001). In our experiments, whether Ret-RGS1 increased or decreased the evoked current also depended on modification of the G-protein: Ret-RGS1 (1 ng RNA) increased evoked current with wild-type $G\alpha_{o1}$ (3 of 3 experiments) but decreased the evoked current with PTX-insensitive $G\alpha_o$ (six of seven experiments). An additional effect of Ret-RGS was to reduce the basal current; this is expected because its GAP activity leaves more $G\alpha$-GDP, which binds $G\beta\gamma$, and thus reduces the free $G\beta\gamma$ available to open the GIRK channel. Because the basal current sets the noise, when Ret-RGS decreases the basal current and increases the evoked current, it increases the signal-to-noise ratio.

Implication of the function of Ret-RGS1 in ON bipolar cells

Light-evoked current in ON bipolar cells is caused by a decrease in glutamate concentration. Therefore, Ret-RGS1 should accelerate the rising phase of the light response (rather than its falling phase). The precise time of activation and deactivation in the *Xenopus* oocyte is obviously different from the light responses of the ON bipolar cell because of huge differences in rates of solution exchange and other intrinsic oocyte factors (Zhang et al., 2002); however, the effect of Ret-RGS1 and the dependence on its amount should be comparable.

Ret-RGS1 is present in both the slow rod bipolar cell and the faster cone bipolar cell; we expect the cone bipolar to express more Ret-RGS1 relative to its $G\alpha_{o1}$. This seems plausible because for photoreceptors, where the rod is slow and the cone is faster, the cone expresses more RGS9 than the rod (Cowan et al., 1998; Zhang et al., 2003). Our current assays, however, cannot quantify these ratios precisely enough to test this.

Under certain conditions, Ret-RGS1 increases the agonist response. It is therefore tempting to speculate that in rod bipolar cells Ret-RGS1 should increase glutamate response. The rod bipolar cell carries the tiny single photon signal from rods to the AII amacrine cell via a high gain system (Ashmore and Falk, 1979; Falk, 1988; Shiells and Falk, 1994). During dark periods, glutamate is released continuously by the rod and hyperpolarizes the rod bipolar cell. If Ret-RGS1 increased the response to glutamate, it would further hyperpolarize the cell and so increase the driving force of the conducting ions. Additionally, a cell maintained at a more hyperpolarized state could give more graded levels of responses at mesopic light levels (twilight). Thus, Ret-RGS1 can increase the single photon response at scotopic luminances and the dynamic range of the rod bipolar cell at mesopic luminances. In short, the location, interaction, and apparent function of Ret-RGS1 are all suited to terminate the mGluR6→$G\alpha_{o1}$ cascade and allow rapid opening of cation channels to light onset. One natural way to test these hypotheses would be to see whether a knock-out slows the ON response. Unfortunately, this experiment is not straightforward because Ret-RGS1 is also expressed in photoreceptor and ON bipolar axon terminals.

Possible functions of Ret-RGS1 in synaptic terminals

Ret-RGS1 is abundant in photoreceptor and rod bipolar synaptic terminals. There, Ret-RGS1 tightly associates with the plasma membrane and also with the synaptic vesicles. Ret-RGS1 shares the property of membrane association with several RGS proteins (RGS9, GAIP, and RGSZ1), but these attach by different mechanisms. Thus, RGS9 attaches via its DEP domain, and both RGSZ1 and GAIP attach via a highly palmitoylated cysteine string motif (De Vries et al., 1996, 1998; Wang et al., 1998; Martemyanov et al., 2003). For Ret-RGS1, the membrane association resists all treatments short of dissolving the membrane. This might be because Ret-RGS1 contains both a cysteine string domain and a putative transmembrane domain that might connect it to the membrane (Faurobert and Hurley, 1997).

With respect to vesicular localization, GAIP is located on clathrin-coated vesicles near the plasma membrane and *trans*-Golgi vesicles (De Vries et al., 1998, 2000). This suggests a role in vesicular trafficking. This was also suggested for RGS-PX1, which serves $G\alpha_s$ and is localized in early endosomes where it contributes to vesicular trafficking (Zheng et al., 2001). By analogy, Ret-RGS1 in synaptic terminals might also serve in vesicle trafficking.

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