Differentially Distributed IP$_3$ Receptors and Ca$^{2+}$ Signaling in Rod Bipolar Cells

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**Purpose.** Inositol (1,4,5)-trisphosphate receptors (IP$_3$Rs) contribute substantially to cytosolic free calcium ion (Ca$^{2+}$) concentration transients and thereby modulate neuronal function. The present study was undertaken to determine the contribution of IP$_3$Rs to the function of rod bipolar cells in the retina.

**Methods.** Immunoreactivity for IP$_3$Rs in rod bipolar cells from mouse retinas was detected by immunocytochemical methods. Intracellular Ca$^{2+}$ concentrations were optically recorded in acutely isolated rod bipolar cells, and biophysical properties of IP$_3$Rs were analyzed with single channel electrophysiology.

**Results.** The distribution of IP$_3$R isoforms was correlated with cytosolic Ca$^{2+}$ transients induced by activation of group I metabotropic glutamate receptors (mGluRs) and with biophysical properties of differentially expressed IP$_3$Rs.

**Conclusions.** The differential distribution of IP$_3$Rs is used by rod bipolar cells to convey Ca$^{2+}$ signals that are distinct in their duration, amplitude, and kinetics at the subcellular level, and that serve the functions of individual subcellular compartments. IP$_3$R-mediated Ca$^{2+}$ signaling indicates a potential mechanism for the adaptation of the ON-pathway of vision and for coincidence and threshold detection in retinal neurons.

Peng et al. have described the localization in the mammalian retina of type 1 IP$_3$R in synaptic terminals of photoreceptors and bipolar cells, as well as in amacrine cell processes, indicating a possible role in neurotransmission. Wang et al. localize type 1 IP$_3$Rs to outer segments of cone photoreceptors and conclude a possible role of IP$_3$Rs in photoreceptor function. In vertebrate retina, IP$_3$Rs had been identified in photoreceptor cells and plexiform layers, horizontal cells, bipolar cells, and Müller glia and had been implicated predominantly in neuronal and Müller cell function. Indirect evidence from a number of studies indicates that IP$_3$Rs potentially play important roles in regulating neuronal function in the retina by affecting physiological processes governed by transients in the intracellular Ca$^{2+}$ concentration.

Bipolar cells are the first interneurons in the glutamatergic vertical pathway of retinal information processing, and integrate signals in the two synaptic layers of the retina. Bipolar cells express group I metabotropic glutamate receptors, a group of G-protein-coupled glutamate receptors, that are coupled to stimulation of PLC and generation of IP$_3$. Rod bipolar cells also have physiologically relevant PLC activity and IP$_3$R-dependent Ca$^{2+}$ stores.

**Materials and Methods.** All experiments described in the present study were carried out in accordance with the appropriate National Institutes of Health and University of North Texas Health Science Center Guidelines for the Welfare, Care and Use of Experimental Animals and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Isolation and Immunohistochemistry of Rod Bipolar Cells.** Rod bipolar cells were isolated from retinas of outbred albino Swiss Webster mice (Harlan, Indianapolis, IN) by mechanical and enzymatic dissociation as described previously, and were identified using morphologic and/or immunocytochemical criteria. Freshly isolated rod bipolar cells, which maintain not only their substructural morphology but also important physiological functional properties during enzymatic and mechanical dissociation were used in the study. To show preservation of subcellular protein distribution after enzymatic and mechanical dissociation of rod bipolar cells, we used stainings of metabotropic glutamate receptors 1 and 5, two upstream elements of IP$_3$-mediated signaling in rod bipolar cells, to verify that the dissociation process did not lead to rearrangements of proteins (see Figs. 2G and H). After plating on coverslips coated with poly-L-lysine, cells were processed for either immunocytochemistry or optical imaging of intracellular Ca$^{2+}$ concentrations. Immunocytochemistry was performed as described previously. Briefly, cells were fixed in 4% paraformaldehyde (w/v) in phosphate buffer and incubated in block-
ing, primary and secondary antibody solutions 2 hours each. Primary antibodies were used at concentrations identified below. Goat anti-mouse or anti-rabbit IgG labeled with Alexa Fluor 488 and Alexa Fluor 594 (diluted 1:500; Molecular Probes, Inc., Eugene, OR) were used to visualize immunoreactivity using standard immunofluorescence microscopy techniques.34–35

For Western blot analyses and isolation of endoplasmic reticulum (ER) from bipolar cells, ON-bipolar cells which comprise both ON-cone and rod bipolar cells of which ON-cone bipolar cells make up approximately 36% and rod bipolar cells approximately 64%36,37 were isolated by immunopanning. After dissociation of retinas, cells were incubated in vials (Corning, Acton, MA) coated with polyclonal antibody to the N-terminal extracellular region of metabotropic glutamate receptor 6 (mGluk6; rabbit polyclonal, 1:500; Novus Biologicals, Littleton, CO). mGlur6-positive ON bipolar cells that adhered to the vials were collected after centrifugation at 500g and used for Western blot analyses (WB) or ER isolation. Immunoreactivity for intracellular Ca2+ channels was assayed with the standard Western blotting technique.1 Primary antibodies were used at concentrations identified below.

Antibodies

Intracellular Ca2+ channels were detected with isoform-specific antibodies: type 1 IP3R (EMD Biosciences, La Jolla, CA) rabbit polyclonal, 1:1000 for WB, 1:100 for immunocytochemistry (ICC)46, type 2 IP3R (EMD Biosciences) rabbit polyclonal, 1:1000 for WB, 1:100 for ICC50, type 3 IP3R (BD Biosciences Pharmingen, San Diego, CA) mouse monoclonal, 1:1000 for WB, 1:100 for ICC46; pan RyR (Affinity BioReagents, Golden, CO) mouse monoclonal, 1:1000 for WB, 1:50 for ICC.46 Additionally, specific antibodies were used to immunolabel mGlur1 (Chemicon, Temecula, CA; rabbit polyclonal, 0.2 μg/ml for ICC)1 mGlur5 (Chemicon; rabbit polyclonal, 1 μg/ml for ICC)1 to isolate ON bipolar cells using mGlur6 immunoreactivity (Novus Biologicals; rabbit polyclonal, 1:500 for immunopanning), and to characterize rod bipolar cells using protein kinase Cα immunoreactivity (Seikagaku, Tokyo, Japan; mouse monoclonal, 1:100 for ICC).59

Optical Imaging of Intracellular Ca2+ Concentrations

Calcium imaging was performed as described previously.54–57 Briefly, after the isolation of retinal neurons, cells were transferred to L-15 medium (Leibovitz medium; Sigma-Aldrich, St. Louis, MO) and were incubated in 4 μM cell permeant fluo-3 (fluor-3-acetoxymethyl-ester; Molecular Probes) at 37°C for 30 minutes, washed, and recorded for up to 6 hours after dissociation. Changes in fluorescence intensity of the Ca2+ indicator fluo-3 in loaded cells were measured over time with time-lapse videomicroscopy (Olympus IX70, Olympus, Japan; Hamamatsu ORCA-ER, Hamamatsu, Japan; Lambda DG-4 Ultra High Speed Wavelength Switcher with appropriate filter sets; Sutter Instrument Company, Novato, CA; SimplePCI Imaging Software v. 5.2; Compix Inc., Imaging Systems/Hamamatsu Photonics Management Corporation, Bridgewater, NJ). Cells received fresh L-15 medium constantly using a gravity-fed perfusion system with a flow rate of 1 ml/min. Images were acquired every 0.5 second and Ca2+ transients were calculated as the ratio of the fluorescence intensity during drug application (F) over the average baseline fluorescence intensity 10 s before drug application (F0). Subcellular regions of interest of rod bipolar cells were analyzed independently and their spatiotemporal patterns of Ca2+ transients were analyzed separately.

During the calcium imaging experiments, the group I metabotropic glutamate receptor agonist (S)-3,5-dihydroxyphenylglycine (SDHPG)34–55 (Sigma-Aldrich) was applied to the imaging chamber at pharmacologically relevant concentrations between 0.1 and 250 μM in a 0.5 seconds bolus application. Similarly, thapsigargin (10 μM; EMD Biosciences), an inhibitor of sarcoplasmic and endoplasmic reticulum Ca2+ ATPases,56 was bath-applied to release Ca2+ from intracellular stores at the end of experiments to test the functionality and Ca2+ levels of intracellular Ca2+ stores. In several experiments, 100 μM dantrolene and/or 1 μM xestospongin C (Sigma-Aldrich) were included in the perfusate to blockryanodine receptors and/or IP3Rs, respectively. To test the dependence of measured Ca2+ transients on the extracellular Ca2+ concentration, Ca2+-free L-15 medium containing 5 mM EGTA to buffer trace amounts of free Ca2+ was used instead of regular L-15 medium. When cells were tested in the Ca2+-free medium environment they were equilibrated in Ca2+-free medium for 2 minutes before the application of mGlur1 agonists. No significant depletion of Ca2+ stores occurred during this time.

Three or more independent experiments analyzing at least 5 cells each were performed to obtain data for each experimental condition, and statistical analyses used standard one-way or multiple ANOVA for comparisons of parametric populations.

Single Channel Electrophysiology

Intracellular calcium channels, present in ER vesicles prepared from ON-bipolar cells that had been isolated with mGlur6 immunopanning, were measured as described previously46–48 after incorporation into planar lipid bilayers. Bilayers had a 250 mM HEPES-Tris solution, pH 7.35 on the cytosolic side and a 250 mM HEPES, 55 mM Ba(OH)2 solution, pH 7.35 on the ER lumen side of the channel. The identity of IP3Rs was verified by activation with its ligand IP3 and inactivation by 50 mg/L heparin or 1 μM xestospongin C. No RyRs were observed in the ER preparations. The activity of IP3Rs was monitored over a range of cytosolic-free Ca2+ and IP3 concentrations. Channel activity was recorded under voltage-clamp conditions, filtered at 3 kHz and digitized using a planar lipid bilayer workstation (Warner Instruments, Inc., Hamden, CT). Data acquisition and analysis were carried out with pClamp version 8.1 (Axon Instruments, Union City, CA) identifying mean dwell times, current amplitudes, and open probability (P0). The identity of IP3 subtypes was determined using known biophysical parameters particularly the channel activity dependence on cytosolic IP3 and free Ca2+ concentrations57–59 (reviewed in Refs. 2, 54, 55). Based on these properties the group of IP3Rs with high IP3 sensitivity was identified as IP3R2 and the group of IP3Rs with intermediate IP3 sensitivity as IP3R1.

The data for each experimental condition were obtained from three or more independent experiments, and statistical analyses used standard one-way or multiple ANOVA for comparisons of parametric populations.

Results

In the present study, the distribution and function of intracellular calcium channel isoforms in rod bipolar cells of the mouse retina were investigated with specific antibodies, optical imaging of intracellular Ca2+ concentrations, and electrophysiology. We identified a correlation between the isoform-specific differential distribution of intracellular calcium channels and their isoform-specific biophysical and cellular Ca2+ signaling properties in neurons. Our results indicated that the differential distribution of intracellular Ca2+ channel isoforms with different biophysical properties can be used by neurons to produce cellular signaling patterns as described for other cell types.47–51 Such functional distribution patterns in neurons potentially provide a basis for compartment-specific intracellular signaling mechanisms that convey temporally and spatially distinct signaling patterns.

In mGlur6-immunopurified ON-bipolar cells (see Materials and Methods), which comprise both ON-cone and rod bipolar cells (approximately 36% and 64%, respectively60–63), Western blot analyses showed the expression of two IP3R isoforms, types 1 and 2 IP3R (Fig. 1). Both antibodies against type 3 IP3R and RyRs showed no significant signals when compared to mouse control tissues (Fig. 1). These results from immunoblotting experiments were confirmed by immunocytochemistry. Immunofluorescence staining of acutely isolated rod bipolar...
cells showed specific label with signals above control levels (Figs. 2A and 2B) only for type 1 and 2 IP3R (Figs. 2C and 2D). The stainings for type 1 IP3R immunoreactivity indicate that type 1 IP3R can be found throughout the entire rod bipolar cell. Highest levels of immunoreactivity, however, were found in somata and axon terminals (Fig. 1C). In contrast, immunoreactivity for type 2 IP3R was restricted to the dendrites and only very faint immunofluorescence label for type 2 IP3R immunoreactivity was found in the distal portion of the rod bipolar cell soma (Fig. 1D).

When rod bipolar cells were stained with antibodies against type 3 IP3R and RyRs that had been used for immunocytochemistry successfully in previous publications,40–41 no specific immunofluorescence signals were detected (Figs. 2E and 2F). However, with immunolabeling of acutely isolated rod bipolar cells we could corroborate findings from a previous study7 that had identified group I mGluRs in rod bipolar cells postsynaptically to rod photoreceptor cells using vertical cryosections of the retina and ultrastructural immunolocalization. Immunoreactivity for mGluR1 and mGluR5, two upstream elements of the mGluR signaling pathway, was found in the somata and axon terminals (Fig. 1C). In contrast, immunoreactivity for type 2 IP3R was restricted to the dendrites of rod bipolar cells. Acutely isolated rod bipolar cells were fixed and stained for type 1 (C), type 2 (D), and type 3 (E) IP3R immunoreactivity, as well as for RyR immunoreactivity (F). Controls include secondary antibody controls for both types of secondary antibodies used [anti-mouse (A); anti-rabbit (B)], as well as labeling of mGluR1 (G) and mGluR5 (H) immunoreactivity. For each panel, differential interference contrast images show the typical rod bipolar cell morphology with dendritic tree, soma axon, and axon terminal system.

**FIGURE 2.** Localization of IP3R and RyR immunoreactivity in mouse rod bipolar cells. Acutely isolated rod bipolar cells were fixed and stained for type 1 (C), type 2 (D), and type 3 (E) IP3R immunoreactivity, as well as for RyR immunoreactivity (F). Controls include secondary antibody controls for both types of secondary antibodies used [anti-mouse (A); anti-rabbit (B)], as well as labeling of mGluR1 (G) and mGluR5 (H) immunoreactivity. For each panel, differential interference contrast images show the typical rod bipolar cell morphology with dendritic tree, soma axon, and axon terminal system.
Ca²⁺ Signaling in Rod Bipolar Cells

In neurons, intracellular Ca²⁺ signaling is critically determined by IP₃R-mediated release of Ca²⁺ from intracellular Ca²⁺ stores. Especially for retinal neurons, the second messenger substance IP₃ has been implicated indirectly in several studies as an important physiological component of intracellular and neuronal signaling. In bipolar cells that integrate signals in the two synaptic layers of the retina, as the first interneurons in the glutamatergic vertical pathway of retinal information processing changes in the intracellular Ca²⁺ concentration have been shown to control neurotransmitter release. Specifically, rod bipolar cells express group I metabotropic glutamate receptors and have physiologically relevant PLC activity and IP₃R dependent Ca²⁺ stores. Therefore, these findings corroborate data related to the immunolocalization of metabotropic glutamate receptors 1 and 5 (Figs. 2G and 2H) indicate that the native subcellular protein distribution was preserved after enzymatic and mechanical dissociation of rod bipolar cells and did not lead to rearrangements of proteins.

These findings corroborated data related to the immunolocalization of type 1 IP₃R (9-11) and expanded these reports to include the localization of type 2 IP₃R. But more importantly, they incorporate functional properties as well as mechanisms of action of IP₃R-mediated Ca²⁺ signaling in rod bipolar cells. Our results also supported the notion that the differential localization of functionally distinct isoforms of intracellular Ca²⁺ channels determines cellular signaling patterns and functions. The results also identify IP₃R-mediated signaling initiated by glutamatergic neurotransmission as a potential mechanism of action in rod bipolar cells. We determined that the differential distribution of IP₃ isoforms influences group 1
mGluR and IP₃-mediated Ca²⁺ signaling in mouse rod bipolar cells and might play a critical role in the modulation of signaling in these first interneurons in the glutamatergic vertical pathway of retinal information processing.

Low agonist and therefore low cytosolic IP₃ concentrations would preferentially activate IP₃R isoforms with a high affinity for IP₃ [52,54, 55] in the dendrites (Figs. 2D, 3B, 4A, 4C, 4D). In contrast, higher agonist and therefore higher cytosolic IP₃ concentrations would also be able to activate IP₃R isoforms with a lower affinity for IP₃ [52,54, 55] that in rod bipolar cells are found throughout the cell, but predominantly in the soma and the axon terminal (Figs. 2C, 3B, 4B-4D). These correlations between localization of receptor isoforms and their functional and biophysical properties resulting in specific Ca²⁺ signaling patterns are further supported by the diffusion properties of IP₃ [56] and spatial constraints of the group I mGluR and IP₃ signaling system (Figs. 2C, 2D, 2G, 2H). Allbritton and colleagues [56] identified IP₃ as a global intracellular messenger measured in cytosolic extracts with a fast diffusion coefficient of 283 μm² and a long, effective range of 24 μm taking diffusion coefficient and lifetime into account. Therefore, even if one assumes that IP₃ is exclusively being generated at the tips of rod bipolar cell dendrites, which is not supported by the group I mGluR immunolocalization data [1] (Figs. 2G, 2H) showing extrasynaptic expression of group I mGluRs, IP₃Rs in the soma would still be activated if they had a similar affinity to IP₃ as IP₃Rs in the dendrite, (i.e., the same IP₃R isoforms in both soma and dendrites). However, the existence of IP₃Rs with different affinities for IP₃ in the soma and the dendrites (Figs. 2C, 2D, 4) allows rod bipolar cells to discriminate the strength of incoming glutamate/IP₃ signals (Fig. 3). Recent studies show that mGluR 1 function in addition to binding of the receptor to L-glutamate also depends on the extracellular calcium concentration. [57–76] The present study used native group I mGluRs as an indirect means of stimulating IP₃ production in rod bipolar cells, allowing not only the control of the amount but more importantly the location of the IP₃ release better than other current pharmacological or cell biological techniques, and at the same time, limiting the observed effects to a ligand-specific interaction and subsequent second-messenger mobilization. Even though high S-DHPG doses used in the present study produced maximal receptor activity, future studies investigating the involvement of changes in extracellular calcium concentrations need to address modulating effects of extracellular calcium concentrations on the results of the present study.

In ON-bipolar cells, glutamate released by photoreceptor cells produces hyperpolarization by binding to mGluR6, which closes a cGMP-gated cation channel on the plasma membrane. [57] As reported previously [58–69] (reviewed in Ref. 61), the cytosolic Ca²⁺ concentration is critical for the regulation of this cation channel and thereby the mGluR6-mediated adaptation of the ON-pathway of visual neurotransmission to changing light levels. Results of the present study potentially indicate that the group I mGluR-mediated effects of glutamate could modulate the mGluR6 pathway through regulation of the cytosolic Ca²⁺ concentration [58–60] (reviewed in Ref. 61), and zero current levels of channel activity and downward deflections indicate channel openings. (A) and (B) Electrophysiological recordings of two representative experiments with three traces at 0.01, 0.1, and 1 μM IP₃, exemplifying the two types of IP₃Rs that could be observed in mouse rod bipolar cells. (C) and (D) summarize the IP₃ dependence of the absolute and normalized open probability (Pₒ) respectively, for the two types of IP₃Rs isolated from mouse rod bipolar cells. Based on the biophysical properties, the group of IP₃Rs with high IP₃ sensitivity is identified as IP₃R2 (type 2 IP₃R; n = 9) and the IP₃R with intermediate IP₃ sensitivity as IP₃R1 (type 1 IP₃R; n = 11).
potentially adapt the sensitivity of rod bipolar cells to changing light levels.

In addition to this potential function of the group I mGluR/IP$_R$R pathway, other reports also support the notion of group I mGluR activation resulting in hyperpolarization via activation of Ca$_{2+}$-activated K$^+$ channels as a result of IP$_R$ mediated Ca$_{2+}$ signaling (reviewed in Ref. 32), which is relevant in light of reported Ca$_{2+}$-activated K$^+$ channel activity in bipolar cells. However, it should be noted that depending on the system and physiological environment, other functions of group I mGluR including neuronal depolarization are also being discussed.32

Similar to processes observed in other portions of the CNS, the expression of a low affinity IP$_3$R in the soma of rod bipolar cells could also serve as a coincidence and threshold detector for elevated levels of IP$_3$, in the cytosol, providing signal integration in the soma. In contrast, the expression of a high affinity IP$_3$R in the dendrites of rod bipolar cells would help to maintain regulation of synaptic events as discussed above for a potential involvement in mGluR-mediated signaling. In summary, rod bipolar cells can use the differential distribution of IP$_3$R isoforms to produce spatio-temporally distinct cytosolic Ca$_{2+}$ signals that contribute to Ca$_{2+}$-dependent functions of subcellular compartments.

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