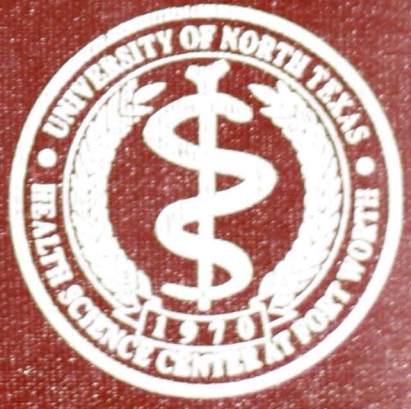
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The retina, a specialized part of the central nervous system (CNS) is the innermost layer of the eye responsible for capturing light and converting that light response into a signal that can be transmitted through the optic nerve and onto the brain for interpretation. The ability of the retina to perceive light is dependent on its sensory neurons and the neural circuitry present that initiate the primary stage of processing the image being visualized, which then transmits an electrical signal down the optic nerve to the brain for processing and ultimately visual perception. In the vertical pathway of the visual process that involves the photoreceptor cells, bipolar cells and the ganglion cells, glutamate is the main excitatory neurotransmitter. Communication between these cells is dependent upon the release of glutamate into the synaptic region within both the outer plexiform layer and inner plexiform layer, a process that is Ca^{2+} regulated.

In neurons, Ca^{2+} regulates a plethora of processes such as gene expression, cell death, synaptic plasticity and neurotransmitter release since it serves as a critical intracellular messenger. In view of the involvement of Ca^{2+} in a variety of physiological processes, it is essential for the intracellular Ca^{2+} concentration to be tightly regulated within neuronal cell. Regulation of Ca^{2+} signaling within retinal neurons can occur via inositol 1,4,5-trisphosphate (IP_3) receptors (IP_3Rs) and ryanodine receptors (RyRs). These receptors are involved in the release of Ca^{2+} from the intracellular stores such as

the endoplasmic reticulum (ER) into the cytosol. IP_3Rs and RyRs contribute substantially to cytosolic free Ca^{2+} concentration transients and thereby play an important role in neuronal function. The purpose of the study was to determine the role of mGluRs, IP_3Rs and RyRs in increasing intracellular Ca^{2+} levels in retinal neurons as related to signaling and neurotransmitter release.

The present study provides experimental evidence for the following mechanisms:

- Activation of mGluR8 in photoreceptor cells reduced cytosolic Ca^{2+} concentration by inhibition of the voltage gated Ca^{2+} channels on the plasma membrane.
- The distribution of IP_3R and RyR isoforms was associated with cytosolic Ca^{2+} transients and the IP_3R induced transients occurs by activation of group I mGluRs.
- In rod bipolar cells, the main increase in cytosolic Ca^{2+} concentrations during depolarization is due to Ca^{2+} release from internal stores via activation of RyR.

The results of the present study contribute to the understanding of intracellular Ca^{2+} signaling in retinal neurons and Ca^{2+} signaling mechanisms. This is of relevance for identifying mechanisms controlling neurotransmitter release and possible pharmacological targets in neurodegenerative retinal diseases characterized by Ca^{2+} dyshomeostasis.

FUNCTION OF DIFFERENTIALLY EXPRESSED INTRACELLULAR CALCIUM CHANNELS IN RETINAL NEURONS

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DISSERTATION

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LIST OF ABBREVIATIONS

AMD	Age Related Macular Degeneration
Ca^{2+}	Calcium
$[Ca^{2+}]_c$	Cytosolic calcium concentration
$[Ca^{2+}]_i$	Intracellular Ca^{2+}
cADPR	Cyclic ADP ribose
cGMP	Cyclic guanosine monophosphate
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate
CICR	Calcium-induced calcium release
CNS	Central nervous system
CNV	Choroidal neovascularization
CTX	cholera toxin
Co-IP	Co-immunoprecipitation
DAG	Diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DSP	Dithiobis-succinimidyl propionate
EGTA	Ethylene glycol bis (2-aminoethyl ether)-N,N,N'N'-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum

GPCR	G protein coupled receptors
IB	Immunoblot
ICC	Immunocytochemistry
iGluR	ionotropic glutamate receptors
INL	Inner nuclear layer
IP	Immunoprecipitation
IP_3	Inositol 1,4,5-trisphosphate
IP_3R	Inositol 1,4,5-trisphosphate receptor
IPL	Inner plexiform layer
L-AP4	L-2-amino-4-phosphonobutyrate
L-SOP	L-serine-*O*-phosphate
mGluR	Metabotropic glutamate receptors
OPL	Outer plexiform layer
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PLC	Phospholipase C
PTX	Pertussis toxin
pCa	Negative decadic logarithm of the calcium ion concentration
RBC	Rod bipolar cell
RGC	Retinal ganglion cells
ROS	Reactive oxygen species

RPE	Retinal pigment epithelium layer
RTK	Receptor tyrosine kinases
RyR	Ryanodine receptor
SERCA	Sarcoplasmic/endoplasmic–reticulum Ca^{2+}-ATPase
S-DHPG	(S)-3,5-dihydroxyphenylglycine
SDS	Sodium dodecyl sulfate
SR	Sarcoplasmic reticulum
TM	Transmembrane
WB	Western blot

CHAPTER I

INTRODUCTION

In the mammalian retina, Ca^{2+} and Ca^{2+} channels are involved in many different processes including the release of the excitatory neurotransmitters glutamate, which plays a very important role in the processing and transmission of visual information. The process of transmission of a visual signal within the vertical pathway involves the photoreceptor cells, the bipolar cells, and then the ganglion cells, and is regulated by glutamate release from synaptic terminals.

It has been shown that IP_3R type 1 are localized within the synaptic terminals of photoreceptor cells, bipolar cells and amacrine cells processes of the mammalian retina (Peng et al., 1991). These findings are a strong indicator that the IP_3Rs and their Ca^{2+} stores may play a pivotal role in neurotransmitter release.

Since Ca^{2+} signaling controls many aspects of neuronal function and behavior, it is essential to understand the functional role of these Ca^{2+} channels since impairment of cellular Ca^{2+} signaling can be associated with visual dysfunction. Although glutamate is the main excitatory neurotransmitter within the retina, it has the potential to being toxic in higher concentrations, and produce pathological changes due to Ca^{2+} dysfunction.

During this study our focus will be on rod bipolar cells and photoreceptor cells. Bipolar cells are the first inter-neurons within the vertical pathway's glutamatergic system, where ON-bipolar cells comprise both ON-cone and rod bipolar cells (Ghosh et al., 2004; Euler and Wässle 1995). Photoreceptor cells are also part of the vertical pathway's glutamatergic system, and are specialized neurons capable of

phototransduction. The glutamatergic system plays a vital role in memory and information processing in the central nervous system, which includes the retina. Bipolar cells are therefore important for the processing of retinal information, and integration of signals between the two synaptic layers of the retina (Brandstätter et al., 1998; Koulen 1999). Within the glutamatergic system, glutamate is the main neurotransmitter and it is also the most prevalent neurotransmitter in the central nervous system. To assimilate glutamate signaling, neurons express either metabotropic and/or ionotropic glutamate receptors.

Vision and the retina

Within the vertical pathway of the mammalian retina, glutamate acts as the main excitatory neurotransmitter. The neurons of the vertical pathway are the photoreceptor cells, the bipolar cells and the ganglion cells. Activation of this process occurs when light travels through the retina to stimulate the rod and cone photoreceptors. Photons are absorbed by the visual pigments within these cells, eliciting a process called phototransduction. This process is then translated into a biochemical signal which initiates an electrical signal that stimulates the bipolar cells, then the ganglion cells within the neuronal pathway.

Within photoreceptors, the phototransduction process occurs in three stages, (1) the activation of the visual pigments when the light stimulus is received, (2) the activation of the cyclic guanosine monophosphate (cGMP) phosphodiesterase that cleaves cGMP, leading to a reduction of cGMP levels and (3) the closing of cGMP-gated

ion channels, leading to the hyperpolarization of the photoreceptors, thus reducing their activity with respect to glutamate release. Hence, in the presence of light, glutamate release from photoreceptor cells is reduced, and in the dark glutamate release increases.

Glutamate release from photoreceptors can then stimulate bipolar cells. Bipolar cells which are the inter-neurons within this system, directly and indirectly relay signals from the photoreceptor cells to the ganglion cells. 11 different types of bipolar cells have been discovered in the human retina using Golgi staining (Boycott and Wässle, 1991; Kolb et al., 1992; Mariani, 1985). 10 of those are designated for cones and one for rods but with rods being the most prevalent cells within the retina, rod bipolar cells are found to exist in the highest numbers.

Bipolar cells belong to either one of two groups, the ON-bipolar and the OFF-bipolar cells depending upon their response to glutamate. ON-bipolar cells are depolarized in response to light conditions which is characterized by low glutamate release from photoreceptors, and hyperpolarized in dark conditions, thus they are considered sign-inverting (+ and -, - and +). OFF-bipolar cells react in an opposite manner in that light conditions elicits a hyperpolarization and dark conditions causes then to depolarize, hence they are considered sign-conserving (+ and +, - and -). When bipolar cells are depolarized they release glutamate onto ganglion cells, and when they are hyperpolarized, there is a reduction in the glutamate being released from their axon terminals onto ganglion cells.

Glutamate and its receptors

Glutamate is the main excitatory neurotransmitter in the vertical pathway of the mammalian retina. Photoreceptors release glutamate unto their postsynaptic partner, the bipolar cells, which also release glutamate unto ganglion cells. Two types of glutamate receptors exist, the ionotropic glutamate receptors (iGluR) which are ligand gated ion channels and the metabotropic glutamate receptors (mGluR) which are G-protein couple receptors. The iGluR are known to play an important role in mediating fast excitatory synaptic transmission (Monaghan et al., 1989; Seeburg et al., 1993; Hollmann et al., 1994), while the mGluRs are slower acting, since they are G-protein coupled and can mediate excitatory and inhibitory behaviors based on the G-protein activated, and the second messenger pathway (Pin et al., 1995; Conn et al., 1997). These G protein coupled receptors (GPCR), are classified where the group I (mGluR1 and 5) are $G_{q/11}$, $G_{i/o}$ and/or G_s coupled (Abe et al., 1992; Aramori et al., 1992; Choi et al., 1996; Hay et al., 1994; Joly et al., 1995; McCool et al., 1997; Pin et al., 1992). $G_{q/11}$ coupled receptors involve phospholipase C (PLC) and intracellular Ca^{2+}, and the group II (mGluR2 and 3) and the group III (mGluR4, 6, 7, and 8) often are negatively coupled to adenylyl cyclase.

Our focus during this study will be Ca^{2+} channels and Ca^{2+} mobilization within the retina, more specifically within photoreceptor cells and bipolar cells. Of particular interest are the metabotropic glutamate receptors, and their direct involvement in modulating Ca^{2+} levels within these neurons. These receptors include the group I mGluRs (mGluR1/5) and the group III mGluRs (mGluR8).

mGluR1/5

mGluR1 and 5 subtypes are situated at the periphery of the postsynaptic region (Lujan et al., 1997) and as stated, belong to the Group I mGluRs that are primarily couple to $G_q/_{11}$ proteins that leads to activation of PLC where activation of PLC results in the release of Ca^{2+} from intracellular stores. Group I mGluR that reside in postsynaptic regions are required for the initiation of different forms of synaptic plasticity and also determines the polarity of synaptic plasticity (Jung et al., 2006). It has also been established that the inositol 1, 4, 5-trisphosphate receptors (IP_3R), and the ryanodine receptor (RyR) were found to be involved in several forms of synaptic plasticity via Ca^{2+} release from the endoplasmic reticulum (ER) (Rose 2001).

mGluR8

Metabotropic glutamate receptor Type 8 (mGluR8) has been identified as a member of the group III mGluRs that can be activated by such agonists as L-2-amino-4-phosphonobutyrate (L-AP4) and L-serine-*O*-phosphate (L-SOP) (Conn et al., 1997; Duvoisin et al., 1995). This receptor is closely related to mGluR4, mGluR7, and mGluR6 (74%, 74%, and 70% identical amino acid residues, respectively) (Duvoisin et al., 1995). Functional localization of mGluRs has been described in the mammalian retina (Brandstätter et al., 1998; Brandstätter et al., 2001) and vertebrate retina (Thoreson et al., 1999). mGluR8 is the most recently discovered member of the mGluR family. It was characterized in a mouse retina cDNA library (Duvoisin et al., 1995) and then in the human genome where there is a possibility that it may have some potential relevance to a

form of retinitis pigmentosa (Scherer et al., 1997). In the mammalian retina, messenger RNA from mGluR8 has been found in all nuclear regions and at elevated levels during ontogenesis by in situ hybridization (Duvoisin et al., 1995) and in ganglion cells by single-cell RT-PCR (Tehrani et al., 2000). We recently described mGluR8a as the first glutamate receptor in terminals of photoreceptors in the mammalian retina (Koulen et al., 2002). On activation, mGluR8a mediates a decrease in the intracellular concentration of Ca^{2+} in photoreceptors and provides an inhibitory feedback loop at photoreceptor synapses in the mammalian retina, possibly modulating and fine-tuning synaptic strength (Koulen et al., 2002). This demonstrates the importance of mGluRs in modulating Ca^{2+} within neurons. Ca^{2+} modulation is very important since Ca^{2+} plays a role in a plethora of different processes within cells.

Calcium

Ca^{2+} is an important intracellular messenger molecule in the body. It has been shown to be involved in cell death, cell growth, cell survival, neurotransmitter release, and a number of different processes (Berridge et al., 1998; Mattson et al., 1998). Neurotransmitter release has been shown to be correlated to Ca^{2+} concentrations in presynaptic nerve regions (Katz et al., 1967; Smith et al., 1988). Intracellular Ca^{2+} concentrations are therefore highly regulated within neuronal cells, where one manner of regulation is via mobilizing Ca^{2+} into and out of the ER. Ca^{2+} is stored in the ER, and the mitochondria which act as another avenue for Ca^{2+} sequestration. The concentration of Ca^{2+} outside cells (1-2 mM) is 10,000 fold greater than cytosolic Ca^{2+} concentrations (20-

200 nM) (Mody and MacDonald, 1995; Putney, 1999). In neurons, there are two main sources of Ca^{2+} involved in initiating Ca^{2+}-dependent processes within the cytosol, Ca^{2+} enters the cytosol via ligand and voltage operated ion channels, and is released from the ER via IP_3R (Berridge, 1993; Berridge et al., 2000) and RyR (Coronado et al., 1994; Meissner, 1994; Shoshan-Barmatz and Ashley, 1998; Sutko and Airey, 1996).

Homeostasis of intracellular Ca^{2+} is maintained via these store houses and receptors and also extends to include other proteins such as sarcoplasmic/endoplasmic–reticulum Ca^{2+}-ATPase (SERCA) pump, Ca^{2+}-ATPase, and Na^{+}/ Ca^{2+} membrane exchangers. SERCA pumps Ca^{2+} back into the ER, while the PMCA (plasma-membrane Ca^{2+} ATPase) pump and Na^{+}/ Ca^{2+} exchanger which resides on the plasma membrane remove Ca^{2+} from inside the cell to extracellular regions. There are also Ca^{2+} binding proteins such as calsequestrin and calreticulin that sequesters Ca^{2+} that is free in the cytosol in the SR/ER lumen (Carafoli and Longoni, 1987; Lytton et al., 1992; Carafoli et al., 1996; Blaustein and Lederer, 1999; Carafoli et al., 2001). Additionally, the Golgi apparatus has been shown to take up Ca^{2+} from the cytosol (Pinto et al., 1998).

The mitochondria have also been shown to sequester Ca^{2+} (Carafoli and Lehninger, 1991; Rizzuto et al., 1993). This occurs via Ca^{2+} uniporters that take up Ca^{2+} using the driving force of the electrical gradient across the mitochondrial membrane (Gunter et al., 1994). This process may potentially serve as a method of rapid removal of cytosolic Ca^{2+} from the cytosol (Nicholls, 1995), or a manner of regulating cytosolic Ca^{2+} dynamics within the cell (Simpson and Russell, 1998).

As mentioned, there are different mechanisms by which cells introduce Ca^{2+} into the cytosol via precise stimuli. One of these mechanisms is via channel opening which is short lived. This brief opening of channels results in concise pulses of Ca^{2+} being generated around the immediate opening of the channel before it diffuses into its surroundings. These concise and localized releases of Ca^{2+} are considered elementary Ca^{2+} signals referred to as Ca^{2+} sparks and puffs (Cheng et al., 1993; Bootman and Berridge, 1995; Yao et al., 1995). These elementary Ca^{2+} signals are capable of stimulating a plethora of cellular processes such as vesicle release, synaptic plasticity, membrane excitability, and mitochondria metabolism.

IP_3 Receptors

IP_3R are activated by IP_3, which is produced by the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) by PLC to a IP_3, and a membrane-bound diacylglycerol (DAG). This scenario occurs in response to the activation of G-protein-coupled receptors (GPCRs) or receptor tyrosine kinases (RTKs). The production of IP_3 then leads to the release of Ca^{2+} from intracellular store from within the endoplasmic reticulum (ER) when IP_3 binds to IP_3Rs. IP_3Rs are predominantly expressed in the ER (Maeda et al., 1991; Ross et al., 1989; Maeda et al., 1990; Koulen at al., 2005) but they have also been shown to be localized with other intracellular membranes associated with the ER such as the nuclear envelope (Koulen et al., 2005; Humbert et al., 1996; Leite et al., 2003).

There exist three distinct IP_3R genes which are differentially expressed throughout the body (Nakagawa et al., 1991). These genes can be further divided due to alternative splicing, thus producing additional forms of each gene. Each IP_3R Type 1, 2, and 3 has a different binding affinity to the ligand IP_3 (Newton et al., 1994), therefore these receptors display different kinetics with respect to Ca^{2+} release when IP_3 binds.

The IP_3R are differentially expressed in different tissues and manifest different properties with respect to sensitivity to Ca^{2+} and ATP and IP_3 affinity. The IP_3 ligand affinity to IP_3R have a potency ranking of $IP_3R2 > IP_3R1 > IP_3R3$ (Miyakawa et al., 1999), and the affinity of the ligands to its receptors are influenced by cytosolic Ca^{2+} concentration (Bezprozvanny et al., 1991; Cardy et al., 1997). The sensitivity of IP_3Rs to Ca^{2+} activation have an order of $IP_3R1 > IP_3R2 > IP_3R3$ (Miyakawa et al., 1999).

IP_3Rs are also consider calcium-induced calcium release channels hence cytosolic Ca^{2+} concentrations does influence the IP_3-mediated Ca^{2+} release (Bezprozvanny et al., 1991; Cardy et al., 1997; Iino et al., 1992). As previously stated, IP_3Rs receptors are Ca^{2+} regulated, and in the presence of high intracellular Ca^{2+} bound to IP_3R1 and IP_3R3 have demonstrated a decreased affinity for IP_3 via individual mechanisms thus demonstrating the differential regulation between these receptors (Cardey et al., 1997). In the presence of Ca^{2+}, IP_3R1 demonstrated a bell-shaped Ca^{2+} response curve in contrast to IP_3R3 which does not, showing that the Ca^{2+} regulation between the IP_3R isoforms is different and dependent on receptor isoform in question (Bezprozvanny et al., 1991; Bezprozvanny et al., 1993; Hagar et al., 1998).

Ryanodine Receptors

Calcium-induced calcium release (CICR) is classically defined as the response of the RyR to local increases in the cytolsolic Ca^{2+} concentration. These receptors acquired that name because of their strong affinity for the plant alkaloid ryanodine. Inhibition of RyR channels occur at high concentrations of ryanodine (Koulen and Thrower, 2001). Administration of ryanodine in low doses (~10 nM), has a propensity to increase the frequency of single RyR channel openings. Long durations of open RyR channel activity and a concurrent decrease in ion conductance has been seen with intermediate doses of ryanodine. Millimolar concentrations of caffeine have also been shown to act as an agonist for RyRs (Koulen and Thrower, 2001).

As previously mentioned, intracellular Ca^{2+} is regulated in part by Ca^{2+} mobilizing in and out of the ER. In some cells, the RyR is another well characterized Ca^{2+} channel that releases Ca^{2+} into the cytosol from the ER in response to local cytosolic Ca^{2+} increases. RyR are Ca^{2+} sensitive channels in that their channel activity is due to activation by Ca^{2+} binding to binding sites on the receptor. One method of RyR activation is by increased Ca^{2+} levels due to influx from the extracellular region and activation of IP_3R activate the receptor. RyR inactivation occurs when the Ca^{2+} levels in the cytosol decrease. In addition to Ca^{2+}, RyR can also be activated by adenine nucleotides and caffeine, and inhibited by Mg^{2+} and ruthenium red (Coronado et al., 1994; Meissner, 1994; Shoshan-Barmatz and Ashley, 1998; Sutko and Airey, 1996).

RyRs associate with a few different proteins such as calmodulin, calsequestrin, FK-506 binding protein, and sorcin that have been also shown to modulate RyR activity

(Meyer et al., 1995). Sorcin has been shown to act as an antagonist of RyR, inhibiting Ca^{2+} release (Lokuta et al., 1997).

Thus far, three isoforms of RyR have been discovered, RyRs Types 1, 2, and 3, and they are encoded by three distinct genes that are widely distributed in different mammalian tissues, such as cardiac tissue (Lai et al., 1992; Meissner, 2004; Otsu et al., 1990), skeletal muscle (Coronado et al., 1994; Meissner, 1994; Shoshan-Barmatz and Ashley, 1998; Sutko and Airey, 1996), brain (Lai et al., 1992; McPherson and Campbell, 1990), and smooth muscle (Herrmann-Frank et al., 1991). It has been established that RyR play important roles within the invertebrate retina in that it contributes to the light adaptation processes (Akopian and Witkovsky, 2002; Akopian et al., 1998; Arnon et al., 1997; and Baumann, 2000). It has also been shown that the RyR is immunolocalized within several retinal neuron cell types in lower vertebrates, (Akopian et al., 1998; Krizaj et al., 2003; Krizaj et al., 2004). Despite the evidence in these studies, they do not address the expression and localization of RyR in the mammalian retina.

Calcium and Glaucoma

Researchers have envisioned that in patients with glaucoma, elevated glutamate levels become toxic when Müller cells that help maintain glutamate within homeostatic levels can no longer efficiently modulate glutamate (Anderson and Hendrickson, 1974). Elevated glutamate within the extracellular regions hyper-stimulates ganglion cell ionotropic glutamate receptors such as NMDA, and AMPA/kainite receptors, which as a consequence, elevate Ca^{2+} and Na^{+} within these cells. Such elevation of these ions can

influence the opening of voltage gated ion channels which can further increase the ion concentration within these neurons. A sustained elevation of Na^+ and especially Ca^{2+} ions can trigger apoptotic events within these cells (Quigley et al., 1979).

It has also been suggested that during ischemic and glaucomatous conditions in the retina, there is a strong potential for elevated Ca^{2+} influx into the soma and axon of retinal ganglion cells (RGCs) (Osborne et al., 1999; Osborne et al., 2003; Osborne et al., 2004; Wood et al., 2003). This Ca^{2+} entry maybe occurring due to malfunctions with sodium/potassium ATPase pumps which can lead to the reversal of the sodium/calcium exchanger on the axonal membrane at regions associated with the pathological damage. It was also suggested that excitotoxic secondary damage may ultimately activate NMDA and AMPA/kainate receptor activity, increasing the levels of intracellular Ca^{2+} and Na^+ within these neurons. The caveat though exists because the effects of excitotoxicity and its role in glaucoma are still ambiguous thus its effect on retinal ganglion cells are not completely understood (Ullian et al., 2004).

Age-related macular degeneration

Age-related macular degeneration (AMD) is the leading cause of visual loss in the United States and developing countries in patients over 60 (Klein et al., 1992; Vingerling., 1995). AMD, as stated in the name, causes dysfunction within the macular region of the retina. Two forms of the diseases exist, the wet and the dry form (O'Shea, 1998; Fine et al., 2000; Gottlieb, 2002). The wet form, also considered the advanced form, occurs when atypical and frail blood vessels develop behind the macular, where the

development of these vessels are referred to as choroidal neovascularization (CNV) (Macular Photocoagulation Study Group, 1991). These leaking vessels cause the accumulation of fluids behind the macular, which disrupts the normal orientation of the macular in the eye. This movement of the macular causes it to become damaged. In the dry form, the light-sensitive photoreceptor cells in the macular slowly break down. It is characterized by the development of drusen, which is made up of small, yellowish deposits that form within the layers of the retina and results in the loss of pigmentation in the retina.

The etiology of AMD remains unknown, but a combination of genetic factors (Klein et al., 1994; Seddon et al., 1997; Ishida et al., 2004) and oxidative stress factors in the retina may influence the pathology of the disease (Beatty et al., 2000; Cai et al., 2000). Oxidative stress was shown to contribute to the development of AMD by the Age-Related Eye Disease Study (AREDS) (AREDS 2001). Other factors that can also influence AMD include the individual's environment, gender, smoking, diet, and nutrition, since some of these factors can induce the generation of elevated reactive oxygen species (ROS) in the retina, that can eventually lead to increased oxidative damage (Seddon et al., 1996; Smith et al., 2001).

Researchers have been able to gain insight into the genetics of AMD by performing experiments involving linkage analyses and candidate gene screening. Experimental procedures have shown that an associations of the ABCA4 gene (Allikmets et al., 2000; Allikmets et al., 1997) and the apolipoprotein E (apoE) gene (Klaver et al., 1998; Souied et al., 1998) potentially exist with AMD. Polymorphisms of the apoE gene

in patients with AMD have shown that the occurrence of the ApoE2 allele is associated with an increased propensity to developing AMD versus the ApoE4 allele which may provide a protective effect (Anderson et al., 2001; Schmidt et al., 2000). ApoE is a cholesterol transport protein that possesses immunomodulatory properties has the ability to initiates a signaling cascade that can increase the production of inositol trisphosphate with mobilization of intracellular Ca^{2+} stores (Misra et al., 2001). This mechanism may potentially elevate Ca^{2+} levels to initiate cell death.

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CHAPTER II

INTERACTION BETWEEN MGLUR8 AND CALCIUM CHANNELS IN PHOTORECEPTORS IS SENSITIVE TO PERTUSSIS TOXIN AND OCCURS VIA G PROTEIN βγ SUBUNIT SIGNALING

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INTERACTION BETWEEN MGLUR8 AND CALCIUM CHANNELS IN PHOTORECEPTORS IS SENSITIVE TO PERTUSSIS TOXIN AND OCCURS VIA G PROTEIN ßγ SUBUNIT SIGNALING

ABSTRACT

PURPOSE. The most recently identified metabotropic glutamate receptor (mGluR), type 8 mGluR (mGluR8), has been identified functionally as a presynaptic autoreceptor in rod photoreceptors. This study analyzed the mechanism of action underlying mGluR8 activity and modulation of the cytosolic Ca^{2+} concentration in mouse photoreceptors.

METHODS. The cytosolic Ca^{2+} concentration of acutely isolated rod photoreceptors was monitored optically with microspectrofluorimetry and in the presence of modulators of G protein activity.

RESULTS. mGluR8 activation by the group III mGluR agonists L-2-amino-4-phosphonobutyrate and L-serine-*O*-phosphate or the physiological ligand L-glutamate produced a decrease in influx of extracellular Ca^{2+} into the cytosol. Pretreatment of isolated rod photoreceptors with the G protein uncoupler suramin or pertussis toxin, which inactivates $G_{i/o/z}$ proteins and G_t protein/transducin, or a G protein ßγ subunit–inhibiting peptide abolished this activity. Preincubation of cells with cholera toxin (CTX), an activator of G_s protein, had no effect.

CONCLUSIONS. These results suggest that the function of mGluR8 of modulating the cytosolic Ca^{2+} concentration and thereby potentially the release of neurotransmitter from rod spherules, the axon terminal systems of rod photoreceptors, is mediated by a pertussis

toxin–sensitive G protein potentially via the ßγ subunit. The absence of G_o and G_z proteins, as reported previously, implies a novel potential interaction between G_{i2} and/or G_t protein/transducin and mGluR8 in photoreceptors. These results have potential implications for the regulatory function and pharmacologic targeting of mGluR8 in photoreceptors.

Introduction

In the mammalian retina, L-glutamate is the major excitatory neurotransmitter and is used in the vertical pathway for processing of visual information by photoreceptor, bipolar, and ganglion cells. The same cells also use glutamatergic neurotransmission to activate modulatory horizontal pathways involving GABAergic and glycinergic interneurons in the inner and outer plexiform layers.[1 2 3 4 5] Effects of L-glutamate are mediated by ionotropic (iGluRs) and metabotropic glutamate receptors (mGluRs) in the central nervous system, including the retina. Both classes of glutamate receptors can be further subdivided into groups based on sequence homology, signaling mechanism, and pharmacologic profile.[6 7 8 9] Among the mGluRs, which have been divided into groups I, II, and III both inhibitory and excitatory functions have been determined that are critically linked to the receptor's synaptic localization (pre- versus postsynaptic) and the selective differential activation of second messenger pathways.[9 10 11 12] mGluRs play critical roles in determining and fine tuning retinal physiology.[13 14 15] The most recently identified mGluR, mGluR8, was cloned from a retinal cDNA library[16] and was identified by chromosomal mapping as potentially relevant in neurodegenerative diseases of the

retina.[17] This finding and the unique pharmacologic profile of mGluR8 with mixed properties of both group II and III mGluRs[18 19 20] together generated interest in the investigation of mGluR8's role in retina physiology.[21] We have identified mGluR8a as the first, and so far the only, glutamate receptor in mammalian photoreceptors localized to the axon terminals.[22 23] mGluR8's role as a presynaptic autoreceptor regulating synaptic strength in terminals of photoreceptors was postulated based on the observed decrease of the cytosolic Ca^{2+} concentration in photoreceptors after mGluR8 activation.[22] However, except for the exclusion of cAMP- or protein-phosphorylation–mediated signaling mechanisms and an indirect regulation of L-type calcium channels, little is known about downstream signaling events after mGluR8 activation that ultimately lead to a modulation of the cytosolic Ca^{2+} concentration.[22] In the present study, we therefore investigated whether mGluR8 activity in rod photoreceptor is G protein dependent and if so which class or type of G protein couples mGluR8 to the decrease in Ca^{2+} influx into the cytosol.

Materials and Methods

Experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and guidelines for the welfare of experimental animals issued by the NIH and the University of North Texas Health Science Center at Fort Worth.

Dissociation of Photoreceptor Cells

Rod photoreceptor cells were mechanically and enzymatically isolated and identified by using morphologic and/or immunochemical criteria, as described previously.[22] Briefly, retinas from adult outbred albino Swiss Webster mice (Harlan, Indianapolis, IN) were dissected and incubated in extracellular solution (ECS; in mM: NaCl, 137; KCl, 5; $CaCl_2$, 2; Na_2HPO_4, 1; $MgSO_4$, 1; HEPES, 10; glucose, 22; pH 7.4) containing 1g/L pronase (P5147; Sigma-Aldrich, St. Louis, MO) and 2 mM EGTA, for 15 minutes at 37°C. After washing the tissue with ECS containing 0.5% bovine serum albumin (BSA), 2 mM EGTA, and 10 mg/L DNase (Sigma-Aldrich) cells were obtained by trituration of the tissue. Isolated cells were plated on poly-D-lysine–coated coverslips and incubated in ECS at 37°C for 30 minutes before the experiments began.

Calcium Imaging

Measurement of intracellular Ca^{2+} concentrations by optical imaging was performed as described previously.[22 24 25] In brief, rod photoreceptor cells were incubated in 2 μM cell-permeant fluorescent dye (Fluo-3-acetoxymethylester; Molecular Probes, Eugene, OR) in ECS at 37°C for 30 minutes and washed with ECS. Intracellular Ca^{2+} concentrations were measured for up to 6 hours after dissociation. Using time-lapse videomicroscopy (model IX70; Olympus, Tokyo Japan; ORCA-ER, a high-resolution 12-bit digital B/W cooled CCD camera, Hamamatsu, Hamamatsu, Japan; Lambda DG-4 Ultra High Speed Wavelength Switcher with appropriate filter sets; Sutter Instrument Co., Novato, CA; SimplePCI Imaging Software, ver. 5.2; Compix Inc., Imaging Systems/Hamamatsu Photonics Management Corp., Bridgewater, NJ) the fluorescence

intensity of the Ca^{2+} indicator dye in loaded cells was recorded over time while cells were constantly perfused with ECS in a gravity-driven perfusion system at a flow rate of 1 mL/min. Ca^{2+} transients were identified as the ratio of the fluorescence intensity during drug application (F) over the average baseline fluorescence intensity 10 seconds before drug application (F/F_0) with an image acquisition rate of 500 ms. Using standard one-way or multiple ANOVA at least three independent experiments with at least five cells each were performed for each experimental condition.

Chemicals Used and Their Application before and during Calcium-Imaging Experiments

mGluR8 agonists (L-2-amino-4 phosphonobutyrate, L-AP4; L-serine-O-phosphate, L-SOP; L-glutamate, L-Glu) were obtained from Axxora, LLC (San Diego, CA), and were bath applied in a bolus application directly into the recording chamber. To analyze the involvement of G proteins in the recorded calcium signals, cells were incubated 2 hours before recording and maintained during recordings in ECS containing G-protein modulators (100 µM suramin, 300 µg/L pertussis toxin [PTX], or 400 µg/L cholera toxin [CTX]; EMD Biosciences, La Jolla, CA).

Peptide Delivery to Dissociated Photoreceptor Cells

To analyze the involvement of the G protein ß1 subunit in the observed calcium-signaling pathway, a 28-mer peptide corresponding to a C-terminal portion of the ß-adrenergic receptor kinase (WKKELRDAYREAQQLVQRVPKMKNKPRS) was

synthesized as its N-terminally acylated and C-terminally amidated form.[26] Dissociated photoreceptor cells were incubated with 100 μM of the 28-mer peptide and a protein-delivery reagent (Chariot; Active Motif, Carlsbad, CA) for two hours at 37°C after the manufacturer's recommendation. After peptide delivery or control incubation with protein delivery reagent alone cells were washed in ECS and used for calcium imaging experiments.

Results

Pharmacology of the mGluR8-Mediated Decrease in Intracellular Ca^{2+} Concentration

As previous studies have shown, mGluR8 is so far the only glutamate receptor found to be expressed by mammalian photoreceptors.[22 23] These studies postulated a role for mGluR8 as a presynaptic autoreceptor that controls Ca^{2+}-dependent neurotransmitter release through modulation of L-type calcium channel activity. However, besides excluding cAMP as a second messenger and an involvement of protein phosphorylation, the identification of the underlying mechanism of action is still lacking. In the present study, we investigated the G-protein pharmacology associated with mGluR8 activity. As in previous studies,[22] we isolated mouse photoreceptors and monitored their intracellular Ca^{2+} concentration with microfluorimetry, in the presence or absence of G protein modulators (Fig. 1). We confirmed previous results[22] showing that mGluR8 activation by either the physiological ligand L-glutamate (Fig. 2a) or the group III mGluR agonists L-AP4 (Fig. 1a, 1b, 1c, 1d, Fig. 2d) or L-SOP (Fig. 2g) resulted in a significant decrease in

the intracellular Ca^{2+} concentration of rod photoreceptors. Figure 1 shows montages of recorded rod photoreceptor cells with their key morphologic features, soma, and inner segment. The outer segment was typically lost, and the axon terminal was typically retracted into the soma (Figs. 1a, 1e). After preincubation with buffer in control experiments, cells responded to an L-AP4 stimulus with a decrease in emission fluorescence intensity correlating to a decrease in intracellular free Ca^{2+} (Figs. 1b, 1c, 1d). However, when cells had been pretreated with PTX, L-AP4-induced activation of mGluR8 had no effect on the intracellular Ca^{2+} concentration of rod photoreceptors (Figs. 1f, 1g, 1h). The effect of PTX was independent of the mGluR8 agonist used and was consistently observed for L-glutamate (Fig. 2b) and L-SOP (Fig. 2h) in addition to L-AP4 (Figs. 1f, 1g, 1h, 2e). Alternatively to PTX, which inactivates $G_{i/o}$ proteins and G_t protein/transducin, we pretreated cells with the G protein uncoupler suramin before stimulating them with mGluR8 agonists. Regardless of the type of agonist used, suramin blocked the effect of mGluR8-mediated reduction of intracellular free Ca^{2+} levels (Figs. 2c, 2f, 2i). Preincubation of rod photoreceptor cells with CTX, an activator of G_s protein had no effect on mGluR8 activity (Table 1).

Mediation of the Effects of mGluR8 by the ßγ Subunit of Heterotrimeric G Proteins

To identify which subunit(s) of the PTX-sensitive heterotrimeric G protein potentially mediate(s) mGluR8 activity, we used G protein ßγ subunit inhibition in isolated rod photoreceptor cells. To this end, we delivered a 28-mer peptide corresponding to a C-terminal portion of the ß-adrenergic receptor kinase (ß-ARK

peptide) into photoreceptors using the protein-delivery reagent (Chariot; Active Motif). ß-ARK peptide has been shown to interact specifically with the ßγ subunit of signal-transducing heterotrimeric G proteins and to block ßγ activity effectively.[26] Peptide delivery of ß-ARK peptide into rod photoreceptor cells abolished mGluR8 activity (Table1). Controls treated with the protein delivery reagent alone showed no effect on mGluR8 activity. In all groups—control, PTX, CTX, suramin, and ß-ARK peptide–treated cells—subsequent exposure to the L-type calcium channel blocker nifedipine still decreases intracellular Ca^{2+} levels, as reported previously,[22] indicating that the use of pharmacologic modulators of G protein function had no direct effect on L-type calcium channel activity.

Discussion

mGluR8 has a unique distribution pattern in the retina,[16 22 23] potential relevance for neurodegenerative diseases of the retina,[17] and a unique pharmacological profile,[18 19 20] all of which make mGluR8 interesting for the investigation of retinal physiology. mGluR8 activity has been associated with the activation of G_i and G_o proteins and the modulation of second-messenger systems.[16 18 27 28] In addition to the coupling to adenylyl cyclase, a regulation of potassium and calcium channel activity by mGluR8 resulting in numerous effects of the receptor in the central nervous system has been reported in several studies.[18 22 28 29 30 31] The present study focused on two related aspects of mGluR8 function, the modulation of the intracellular free Ca^{2+} levels and negative feedback signaling to regulate neurotransmitter release presynaptically.[22 28 32 33 34] The latter

process being Ca^{2+}-dependent ties in directly with the modulation of the intracellular Ca^{2+} concentration by mGluR8 through inhibition of plasma membrane calcium channel activity. The results of the present study support a mechanism of action for mGluR8 potentially using the ßγ subunit of signal-transducing heterotrimeric G proteins.

We explored pharmacologic characteristics of mGluR8 in rod photoreceptor cells using pharmacologic tools for G protein signaling. With our findings that the mGluR8-mediated reduction of intracellular free Ca^{2+} levels is PTX sensitive and CTX insensitive, the question arises as to which signal-transducing heterotrimeric G proteins mGluR8 potentially couples to exert its effects on the intracellular Ca^{2+} concentration in rod photoreceptors. Several studies indirectly indicate that of the PTX-sensitive G_i family of G proteins, G_o,[35 36 37 38 39] and G_z[40] are not expressed by photoreceptors. There is the possibility that photoreceptors express the PTX-sensitive G_{i2} but not G_{i1} or G_{i3}.[41] Even though the immunolocalization data presented in Oguni et al.[41] do not conclusively prove expression of G_{i2} by photoreceptor cells, such an expression combined with data presented in the present study suggests a potential role for G_{i2} in mediating mGluR8's effects. Of the other G proteins that have been described in photoreceptors, G_t/transducin[42 43] and G_{11},[44] only G_t is PTX sensitive[45 46] and represents a potential signaling partner of mGluR8 using a PTX-sensitive and CTX-insensitive signaling pathway, as shown in the present study. This opens up the possibility that G_t/transducin, besides its major function in photoreceptor outer segments,[42 43] may also have a secondary function in photoreceptor somata and the outer plexiform layer. Diurnal

changes in G_t/transducin expression levels that have been reported for G_t/transducin's function in phototransduction[47 48] could therefore also affect mGluR8 signaling.

Indirect physiological evidence indicates the involvement of PTX-sensitive G proteins in the signaling mediated by neurotransmitters other than L-glutamate systems in vertebrate photoreceptors.[49 50 51] In a related study, activation of glutamate transporters in vertebrate rod photoreceptors was able to inhibit presynaptic Ca^{2+} currents.[52] In the same study,[52] no effect on mGluRs was detected, which is potentially due to species differences in receptor pharmacology and/or distribution.

The second finding of the present study, the involvement of the ßγ subunit of signal-transducing heterotrimeric G proteins in the mGluR8-mediated reduction of intracellular Ca^{2+} levels, is supported by evidence from several studies identifying Gα-independent signaling pathways. G protein–gated inwardly rectifying K^+ (GIRK) channels,[53] several voltage-dependent calcium channels,[54 55 56 57] and the fusion machinery of vesicular release[58] have been shown to be direct targets of Gßγ activity. With the effects of Gßγ on the fusion machinery of vesicular release being a calcium-independent process[58] and the clear dependence of mGluR8 activity in rod photoreceptors on extracellular calcium,[22] Gßγ's effects potentially target voltage-dependent calcium channels directly or GIRK channels, thereby indirectly influencing voltage-dependent calcium channels. Expression of voltage-dependent calcium channels in photoreceptors has been described, and predominantly L-type channels had been identified.[59 60 61] In a recently published study, the interdependence of intracellular Ca^{2+} concentration, plasma membrane depolarization, and neurotransmitter vesicle release kinetics were studied in

rod photoreceptors.[62] In this study, the direct coupling of intracellular Ca^{2+} concentration to plasma membrane depolarization[62] expands the physiological relevance of L-type calcium channels in rod photoreceptors.[59 60 61 63 64] However, despite the strong evidence for L-type calcium channels as the source of the standing dark current of Ca^{2+} into the cytosol from the extracellular medium, the involvement of other sources cannot be excluded.[22]

Even though the expression of GIRK channels has not been shown directly, the presence of inwardly rectifying K^+ currents in rod photoreceptors[63 64] allows for a potential involvement of Gßγ-regulated GIRK channel activity in the mGluR8-mediated regulation of intracellular free-Ca^{2+} levels in rod photoreceptors. The exact identity of the Gßγ subunits involved has yet to be determined, but, based on previous studies, potentially involves $Gß_1$ and $Gγ_1$ in rod photoreceptors[65] and may involve different subunits in cone photoreceptors.[65 66 67] Even though the results from the experiments using the G protein ßγ subunit inhibiting (ß-ARK) peptide are conclusive, an involvement of G_i family α subunits cannot be excluded because of a lack of inhibitors that selectively inhibit G_i family α but not ßγ subunits.

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Figure Legend

FIGURE 1

The decrease in the intracellular Ca^{2+} concentrations of rod photoreceptor cells induced by application of L-AP4, a group-III mGluR agonist, was PTX sensitive. Ca^{2+} transients in isolated mouse rod photoreceptor cells ester loaded with the Ca^{2+}-indicator dye fluo-3 were measured optically with time-lapse video microscopy by monitoring changes in the intensity of the fluorescent tracer's emission wavelength maximum. Montages of recordings from two representative experiments are displayed with (**a–d**) showing control preincubated cells and (**e–h**) showing cells that had been preincubated with PTX. Selected images visualize the intracellular Ca^{2+} concentration at (time point: 0 seconds; **b**, **f**), during (time point: 5 seconds; **c**, **g**), and after (time point: 20 seconds; **d**, **h**) application of L-AP4. Whereas in control cells (**b–d**) the high intracellular Ca^{2+} concentration drops significantly after stimulation with the mGluR8 agonist, the same application of L-AP4 has no effect in cells pretreated with PTX (**f–h**). All cells of the control and experimental groups showed a constant low-level decrease in fluorescence intensity due to bleaching of the indicator dye that is not stimulus related. (**a**, **e**) Differential interference contrast (DIC) images of the cells indicating their morphology. *Arrows*: selected typical rod photoreceptor cells. Scale bars, 10 μm.

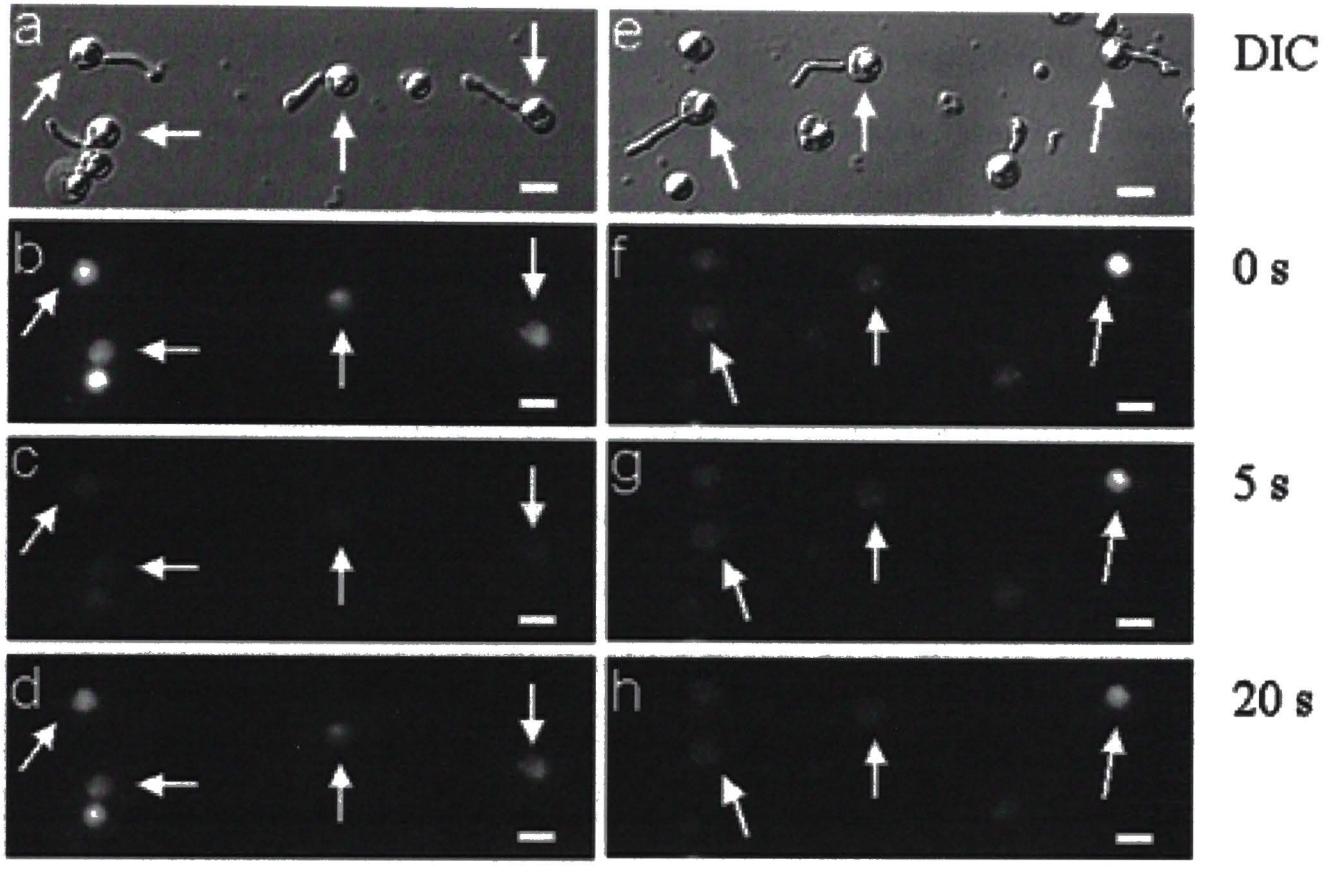
a
e
DIC
b
f
0 s
c
g
5 s
d
h
20 s
control
PTX

FIGURE 2

The mGluR8 mediated decrease in the intracellular Ca^{2+} concentration of isolated rod photoreceptor cells was sensitive to G protein modulation. Traces of individual representative experiments from control cells (**a**, **d**, **g**) and cells that had been pretreated with PTX (**b**, **e**, **h**) or suramin (**c**, **f**, **i**) are shown. The fluorescence intensity of the Ca^{2+} indicator dye was corrected for bleaching and normalized as fluorescence intensity over baseline fluorescence intensity (F/F_0) as a measure of changes in the intracellular Ca^{2+} concentration over time. Single bolus applications (*arrows*) of mGluR8 the agonists L-glutamate (Glu; **a–c**), L-AP4 (**d–f**), and L-SOP (**g–i**) lead to significant reduction of the intracellular Ca^{2+} concentration of isolated rod photoreceptor cells (**a**, **d**, **g**). In contrast, application of the same stimulus has no effect on cells pretreated with PTX (**b**, **e**, **h**) or suramin (**c**, **f**, **i**) during and after drug application.

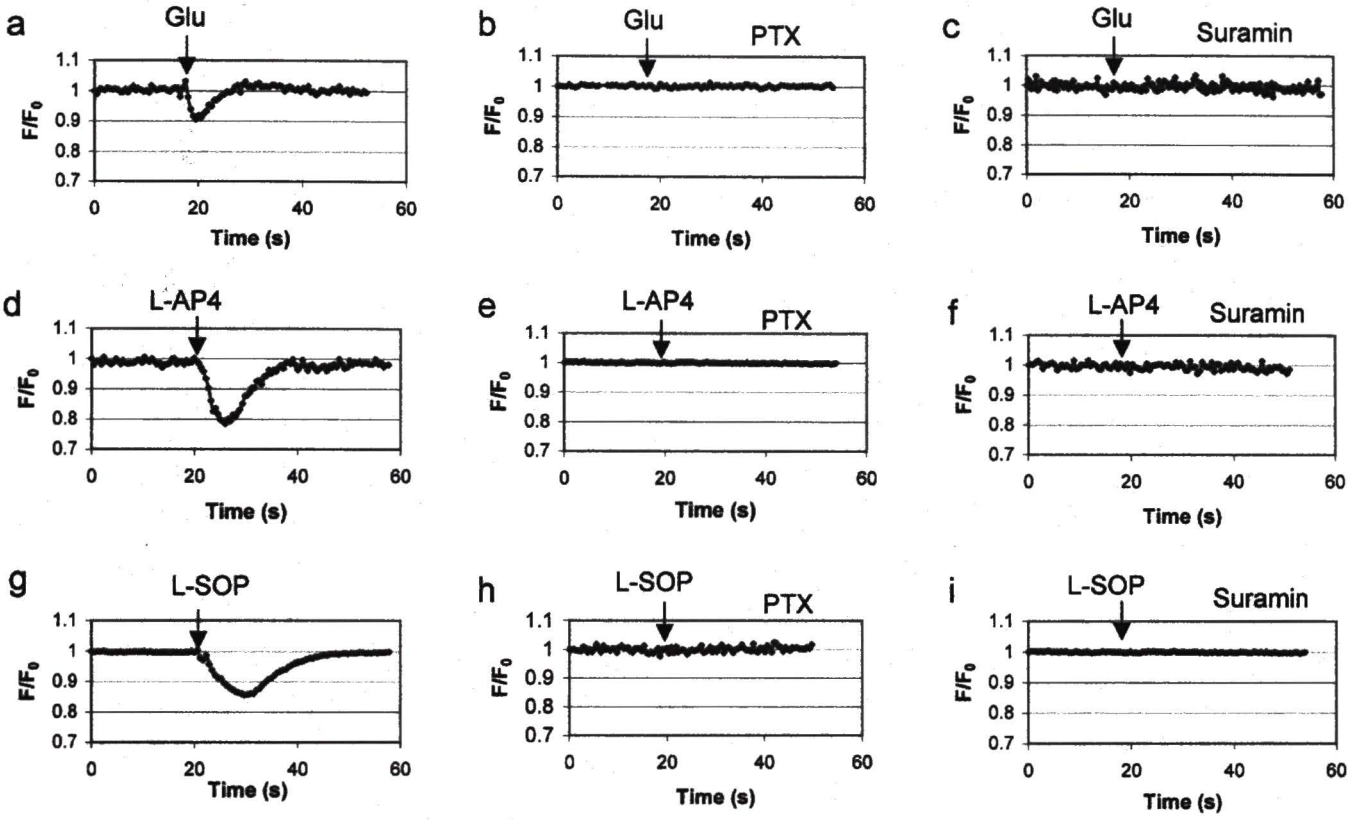
a
Glu
b
Glu
PTX
c
Glu
Suramin
d
L-AP4
e
L-AP4
PTX
f
L-AP4
Suramin
g
L-SOP
h
L-SOP
PTX
i
L-SOP
Suramin
F/F0
1.1
1
0.9
0.8
0.7
0
20
40
60
Time (s)

TABLE 1. Summary of the Pharmacologic Modulation of mGluR8 Responses in Rod Photoreceptor Cells

Drug	*n*	F/F_0 (%)	SEM (%)	SL
L-Glutamate	16	88	7	*
Suramin/Glu	16	102	2	NS
PTX/Glu	16	102	3	NS
CTX/Glu	19	90	6	*
β-ARK peptide/Glu	18	101	1	NS
L-SOP	30	91	4	*
Suramin/L-SOP	20	101	1	NS
PTX/L-SOP	21	100	1	NS
CTX/L-SOP	17	92	3	*
β-ARK peptide/L-SOP	16	100	2	NS
L-AP4	33	89	4	*
Suramin/L-AP4	24	100	1	NS
PTX/L-AP4	28	101	1	NS
CTX/L-AP4	25	88	5	*
β-ARK peptide/L-AP4	19	99	2	NS

Listed are results of three sets of experiments using the mGluR8 agonists, L-glutamate (Glu), L-AP4, and L-SOP. For each of the agonists, cells had either been preincubated as controls or with suramin, pertussis toxin (PTX), cholera toxin (CTX), or a 28-mer peptide corresponding to a C-terminal portion of the β-adrenergic receptor kinase (β-ARK peptide) together with a protein-delivery reagent. For each agonist, a significant decrease in the intracellular Ca^{2+} concentration (significance level [SL], 0.1%: *) compared with control stimulation was observed. Whereas preincubation of cells with CTX did not affect this mGluR8-mediated decrease in the intracellular Ca^{2+} concentration, all other G protein modulators used abolished the observed effect. NS, not significant.

CHAPTER III

DIFFERENTIAL DISTRIBUTION OF INOSITOL (1,4,5)-TRISPHOSPHATE RECEPTOR CALCIUM SIGNALING IN ROD BIPOLAR CELLS

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DIFFERENTIAL DISTRIBUTION OF INOSITOL (1,4,5)-TRISPHOSPHATE RECEPTOR CALCIUM SIGNALING IN ROD BIPOLAR CELLS

ABSTRACT

PURPOSE. Inositol (1,4,5)-trisphosphate receptors (IP_3Rs) 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_3Rs to the function of rod bipolar cells in the retina.

METHODS. Immunoreactivity for IP_3Rs 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_3Rs were analyzed with single channel electrophysiology.

RESULTS. The distribution of IP_3R 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_3Rs.

CONCLUSIONS. The differential distribution of IP_3Rs 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_3R-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.

Introduction

In many neurons, transients in the intracellular Ca^{2+} concentration mediated by release of Ca^{2+} from intracellular stores functionally depend on the activity of inositol 1,4,5-trisphosphate (IP_3)-gated Ca^{2+} channels/ IP_3 receptors (IP_3R). Production of IP_3 through the stimulation of plasma membrane receptors and activation of the phospholipase C (PLC) pathway leads to the activation of IP_3Rs that release Ca^{2+} from intracellular Ca^{2+} stores.[2] This second messenger signaling system has effects on global Ca^{2+} signaling as well as on local, spatially defined Ca^{2+} signals mediating a variety of physiological processes in many cells and particularly in neurons.[3 4 5 6]

Peng et al.[7] have described the localization in the mammalian retina of type 1 IP_3R in synaptic terminals of photoreceptors and bipolar cells, as well as in amacrine cell processes, indicating a possible role in neurotransmission. Wang et al.[8] localize type 1 IP_3Rs to outer segments of cone photoreceptors and conclude a possible role of IP_3Rs in photoreceptor function. In vertebrate retina, IP_3Rs had been identified in photoreceptor cells and plexiform layers,[9] horizontal cells, bipolar cells, and Müller glia[10] and had been implicated predominantly in neuronal and Müller cell function.[11] Indirect evidence from a number of studies indicates that IP_3Rs potentially play important roles in regulating neuronal function in the retina by affecting physiological processes governed by transients in the intracellular Ca^{2+} concentration.[12 13 14 15 16 17 18 19 20]

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.[21 22] Besides evidence for the presence of type 1 IP_3R in these cells[7 8 9 10 11] and

modulation of neurotransmitter release by transients in the intracellular Ca^{2+} concentration,[23 24 25 26 27 28 29 30] the function of intracellular Ca^{2+} channels in bipolar cell physiology, especially in the outer retina, remains elusive. The present study addresses this issue by investigating the distribution of the different types of intracellular Ca^{2+} channels and by analyzing IP_3R-mediated Ca^{2+} signaling and biophysical properties of individual IP_3Rs in an anatomically and physiologically well-characterized type of bipolar cells, rod bipolar cells. These cells express group I metabotropic glutamate receptors,[1 31] a group of G-protein-coupled glutamate receptors, that are coupled to stimulation of PLC and generation of IP_3[32] . Rod bipolar cells also have physiologically relevant PLC activity[33] and IP_3R-dependent Ca^{2+} stores.[7 9 10 11]

Keywords

IP3, intracellular calcium, calcium imaging, ryanodine receptor, metabotropic glutamate receptor, mGluR1, mGluR5

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 Immunochemistry 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 immunochemical criteria.[34 35] Freshly isolated rod bipolar cells, which maintain not only their substructural morphology but also important physiological functional properties during enzymatic and mechanical dissociation,[68 69 70 71 72 73] 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_3R-mediated signaling in rod bipolar cells, to verify that the dissociation process did not lead to rearrangements of proteins (see Figs. 2G and 2H). After plating on coverslips coated with poly-D-lysine, cells were processed for either immunocytochemistry or optical imaging of intracellular Ca^{2+} concentrations. Immunocytochemistry was performed as described previously.[34 35] Briefly, cells were fixed in 4% para-formaldehyde (w/v) in phosphate buffer and incubated in blocking, 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 (mGluR6; 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 Ca^{2+} channels was assayed with the standard Western blotting technique.[1] Primary antibodies were used at concentrations identified below.

Antibodies

Intracellular Ca^{2+} channels were detected with isoform-specific antibodies: type 1 IP_3R (EMD Biosciences, La Jolla, CA) rabbit polyclonal, 1:1000 for WB, 1:100 for immunocytochemistry (ICC)[38] ; type 2 IP_3R (EMD Biosciences) rabbit polyclonal, 1:1000 for WB, 1:100 for ICC[39] ; type 3 IP_3R (BD Biosciences Pharmingen, San Diego, CA) mouse monoclonal, 1:1000 for WB, 1:100 for ICC[40] ; pan RyR (Affinity BioReagents, Golden, CO) mouse monoclonal, 1:1000 for WB, 1:50 for ICC.[41] 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 $C\alpha$ immunoreactivity (Seikagaku, Tokyo, Japan; mouse monoclonal, 1:100 for ICC).[35]

Optical Imaging of Intracellular Ca^{2+} Concentrations

Calcium imaging was performed as described previously.[34 42 43] 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 (fluo-3-acetoxymethylester; Molecular Probes) at 37°C for 30 minutes, washed, and recorded for up to 6 hours after dissociation. Changes in fluorescence intensity of the Ca^{2+} 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 Ca^{2+} 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 (F/F_0). Subcellular regions of interest of rod bipolar cells were analyzed independently and their spatiotemporal patterns of Ca^{2+} transients were analyzed separately.

During the calcium imaging experiments, the group I metabotropic glutamate receptor agonist (S)-3,5-dihydroxyphenylglycine (S-DHPG)[44 45] (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 Ca^{2+} ATPases,[46]

was bath-applied to release Ca^{2+} from intracellular stores at the end of experiments to test the functionality and Ca^{2+} levels of intracellular Ca^{2+} stores. In several experiments, 100 μM dantrolene and/or 1 μM xestospongin C (Sigma-Aldrich) were included in the perfusate to block ryanodine receptors and/or IP_3Rs, respectively. To test the dependence of measured Ca^{2+} transients on the extracellular Ca^{2+} concentration, Ca^{2+}-free L-15 medium containing 5 mM EGTA to buffer trace amounts of free Ca^{2+} was used instead of regular L-15 medium. When cells were tested in the Ca^{2+}-free medium environment they were equilibrated in Ca^{2+}-free medium for 2 minutes before the application of mGluR1 agonists. No significant depletion of Ca^{2+} 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 previously[42 43] 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 IP_3Rs was verified by activation with its ligand IP_3 and inactivation by 50 mg/L heparin or 1 μM xestospongin C. No RyRs were observed in the ER preparations. The activity of IP_3Rs was monitored over a range of cytosolic-free Ca^{2+} and IP_3 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 (P_o). The identity of IP_3R subtypes was determined using known biophysical parameters particularly the channel activity dependence on cytosolic IP_3 and free Ca^{2+} concentrations[52 53] (reviewed in Refs. [2], [54], [55]). Based on these properties the group of IP_3Rs with high IP_3 sensitivity was identified as IP_3R2 and the group of IP_3Rs with intermediate IP_3 sensitivity as IP_3R1.

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 Ca^{2+} concentrations, and electrophysiology. We identified a correlation between the isoform-specific differential distribution of intracellular calcium channels and their isoform-specific biophysical and cellular Ca^{2+} signaling properties in neurons. Our results indicated that the differential distribution of intracellular Ca^{2+} channel isoforms with different biophysical properties can be used by neurons to produce cellular signaling patterns as described for other cell types.[47 48 49 50 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%, respectively[36 37]), Western blot analyses showed the expression of two IP_3R isoforms, types 1 and 2 IP_3R (Fig. 1) . Both antibodies against type 3 IP_3R 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 IP_3R (Figs. 2C and 2D) . The stainings for type 1 IP_3R immunoreactivity indicate that type 1 IP_3R 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 IP_3R was restricted to the dendrites and only very faint immunofluorescence label for type 2 IP_3R 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 IP_3R 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 study[1] 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 IP_3R-mediated signaling in rod bipolar cells, was found in the dendrites and distal one-quarter to one-third of rod bipolar cell somata (Figs. 2G and 2H) , consistent with synaptic release sites for the ligand of group I mGluRs, glutamate, by rod photoreceptor cells in the outer plexiform layer[1] and, at the same time, indicating the major sites of IP_3 generation in the distal portion of rod bipolar cells.

Based on these findings of the expression of specific isoforms of intracellular Ca^{2+} channels by rod bipolar cells (Fig. 1) and of the differential subcellular distribution of these isoforms (Fig. 2) , we hypothesized that stimulation of IP_3Rs would produce functionally different Ca^{2+} signaling patterns dependent on the amount of IP_3 generated by group I mGluR activation.

Intracellular Ca^{2+} concentrations were recorded optically in rod bipolar cells after ester loading with fluo-3 in the absence of extracellular Ca^{2+}. Changes in fluo-3 emission wavelength maximum as an indicator of changes in intracellular calcium concentrations were monitored. Fluorescence intensity of the Ca^{2+} indicator dye fluo-3 was normalized to the baseline intensity and was used as a measure of relative changes in the intracellular Ca^{2+} concentration and displayed as intensity change over time. Freshly isolated rod bipolar cells were stimulated with bolus applications of a specific agonist of group I mGluR, S-DHPG, to induce IP_3 generation in the distal portion of rod bipolar cells (Fig. 3) . Figure 3A shows an image montage of a representative rod bipolar cell response to S-DHPG stimulation. Low concentrations of the IP_3-generating agonist (0.1–10 μM S-

DHPG) produced a temporally and spatially well-defined increase in the cytosolic Ca^{2+} concentration that was restricted to the dendrites (Fig. 3B , blue trace). The mean maximum amplitude at 10 μM S-DHPG was 9 ± 3% with an average signal duration of 7 ± 3 seconds (mean ± SEM; n = 17). No signals were observed in the soma at low agonist concentrations. At higher agonist concentrations (50–250 μM S-DHPG), Ca^{2+} transients with distinct spatial and temporal characteristics could be observed in dendrites and soma (Figs. 3A and 3B) . Whereas Ca^{2+} transients in the dendrites immediately followed the stimulus (Fig. 3B , blue trace), somata showed a delayed onset response of 22 ± 4 seconds (mean ± SEM; n = 17; Fig. 3B , red trace). Ca^{2+} concentrations in both compartments returned to baseline levels during constant perfusion of the cell with medium. The mean maximum amplitude at 100 μM S-DHPG in the dendrites was 11 ± 4% with an average signal duration of 82 ± 7 seconds (mean ± SEM; n = 16). In the soma, mean maximum amplitude at 100 μM S-DHPG was 15 ± 3% with an average signal duration of 104 ± 9 seconds (mean ± SEM; n = 16). All measurements were obtained in Ca^{2+}-free medium excluding a contribution of ligand- or store-operated Ca^{2+} channels located on the plasma membrane to the Ca^{2+} transients. The absence of the contribution of extracellular Ca^{2+} to the cytosolic Ca^{2+} transients through Ca^{2+} channels of the plasma membrane and the receptor-specific stimulation of group I mGluRs enabled us to focus on the spatio-temporal patterns of IP_3-induced intracellular Ca^{2+} release via mGluR1 in rod bipolar cells.

Based on our findings from the immunochemistry experiments, we hypothesized that the observed Ca^{2+} transients induced by group I mGluR stimulation are mediated by

IP_3R and not RyR. Preincubation and perfusion of rod bipolar cells for 5 minutes before and during group I mGluR stimulation with 100 μM dantrolene to inactivate RyRs[2] did not alter signal amplitude or duration, whereas addition of 1 μM xestospongin C[2] to the perfusion medium for 5 minutes before and during S-DHPG application completely abolished agonist-induced Ca^{2+} transients. Based on the same findings from the immunochemistry experiments, we further hypothesized the existence of IP_3Rs with distinct biophysical properties in rod bipolar cells that can be attributed to the differential expression of both types 1 and 2 IP_3R. To test this hypothesis, we analyzed single channel electrophysiological properties of intracellular calcium channels isolated from mGluR6-immunopurified ON-bipolar cells. Intracellular calcium channels with RyR characteristics (activation by Ca^{2+}, cyclic ADP ribose, caffeine, and block by ryanodine, ruthenium red)[2] were not observed, whereas two populations of IP_3R distinct in their activity dependence on IP_3 concentrations were identified (Fig. 4) . A range of physiologically relevant concentrations of IP_3 on the cytoplasmic side of the channel was tested to evaluate activation of the channels by their ligand. Figures 4A and 4B show representative recordings from IP_3Rs with high and intermediate IP_3 sensitivity, respectively. Dose–response analyses showed that IP_3Rs with high IP_3 sensitivity had an EC_{50} of 63 ± 4 nM and IP_3Rs with an intermediate IP_3 sensitivity had an EC_{50} of 196 ± 7 nM. Based on previously published data on the IP_3 dependence of IP_3Rs,[52 53] reviewed in Refs. [54] , [55] , we tentatively identified the group of IP_3Rs with high IP_3 sensitivity as IP_3R2 and the group of IP_3Rs with intermediate IP_3 sensitivity as IP_3R1 (Figs. 4C and 4D).

Discussion

In neurons, intracellular Ca^{2+} signaling is critically determined by IP_3R-mediated release of Ca^{2+} from intracellular Ca^{2+} stores.[4] Especially for retinal neurons, the second messenger substance IP_3 has been implicated indirectly in several studies as an important physiological component of intracellular and neuronal signaling.[12 13 14 15 16 17 18 19 20] In bipolar cells that integrate signals in the two synaptic layers of the retina,[21 22] as the first interneurons in the glutamatergic vertical pathway of retinal information processing changes in the intracellular Ca^{2+} concentration have been shown to control neurotransmitter release.[23 24 25 26 27 28 29 30] Specifically, rod bipolar cells express group I metabotropic glutamate receptors,[1 31] and have physiologically relevant PLC activity[33] and IP_3R dependent Ca^{2+} stores.[7 9 10 11] Therefore, these findings of IP_3-mediated Ca^{2+} signaling via group I mGluRs in rod bipolar cells set the stage for experimental analyses presented in the present study. Our findings indicated that, depending on the strength of glutamatergic input, more specifically group I mGluR activation, Ca^{2+} transients with differential varying temporal and spatial properties ensue. Using immunoblotting, immunocytochemistry, optical imaging of Ca^{2+} concentrations, and single channel electrophysiology, we identified the presence and differential distribution of types 1 and 2 IP_3R and potentially excluded the functional expression of other isoforms of intracellular Ca^{2+} channels in rod bipolar cells.

Freshly isolated rod bipolar cells maintain not only their substructural morphology but also important physiological functional properties during enzymatic and mechanical dissociation.[68 69 70 71 72 73] Therefore, they were used as model systems in the present

study to allow a detailed analysis of IP_3R localization and function in mammalian rod bipolar cells. Control experiments using the immunolocalization of metabotropic glutamate receptors 1 and 5 (Figs. 2G and 2H) indicate that the native subcellular protein distribution[1 69] 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 IP_3R[7 9 10 11] and expanded these reports to include the localization of type 2 IP_3R. But more importantly, they incorporate functional properties as well as mechanisms of action of IP_3R-mediated Ca^{2+} signaling in rod bipolar cells. Our results also supported the notion that the differential localization of functionally distinct isoforms of intracellular Ca^{2+} channels determines cellular signaling patterns and functions.[47 48 49 50 51] The results also identify IP_3R-mediated signaling initiated by glutamatergic neurotransmission as a potential mechanism of action in rod bipolar cells. We determined that the differential distribution of IP_3R isoforms influences group I mGluR and IP_3-mediated Ca^{2+} 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 IP_3 concentrations would preferentially activate IP_3R isoforms with a high affinity for IP_3[52 54 55] in the dendrites (Figs. 2D 3B 4A 4C 4D) . In contrast, higher agonist and therefore higher cytosolic IP_3 concentrations would also be able to activate IP_3R isoforms with a lower affinity for IP_3,[52 54 55] that in rod bipolar cells are found throughout the cell, but predominantly in the soma and the

axon terminal (Figs. 2C 3 4B 4C 4D). These correlations between localization of receptor isoforms and their functional and biophysical properties resulting in specific Ca^{2+} signaling patterns are further supported by the diffusion properties of IP_3[56] and spatial constraints of the group I mGluR[1] and IP_3R signaling system (Figs. 2C 2D 2G 2H) . Allbritton and colleagues[56] identified IP_3 as a global intracellular messenger measured in cytosolic extracts with a fast diffusion coefficient of 283 μm^2 and a long, effective range of 24 μm taking diffusion coefficient and lifetime into account. Therefore, even if one assumes that IP_3 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, IP_3Rs in the soma would still be activated if they had a similar affinity to IP_3 as IP_3Rs in the dendrite, (i.e., the same IP_3R isoforms in both soma and dendrites). However, the existence of IP_3Rs with different affinities for IP_3 in the soma and the dendrites (Figs. 2C 2D 4) allows rod bipolar cells to discriminate the strength of incoming glutamate/IP_3 signals (Fig. 3) . Recent studies show that mGluR1 function in addition to binding of the receptor to L-glutamate also depends on the extracellular calcium concentration.[74 75 76] The present study used native group I mGluRs as an indirect means of stimulating IP_3 production in rod bipolar cells, allowing not only the control of the amount but more importantly the location of the IP_3 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 59 60] (reviewed in Ref. [61]), the cytosolic Ca^{2+} 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 Ca^{2+} concentration[58 59 60] (reviewed in Ref. [61]), and potentially adapt the sensitivity of rod bipolar cells to changing light levels.

In addition to this potential function of the group I mGluR/IP_3R 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_3R mediated Ca^{2+} signaling[62 63] (reviewed in Ref. [32]), which is relevant in light of reported Ca^{2+}-activated K^+ channel activity in bipolar cells.[64] 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,[65 66 67] the expression of a low affinity IP_3R 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_3R in the dendrites

of rod bipolar cells would help to maintain regulation of synaptic events as discussed above for a potential involvement in mGluR6-mediated signaling.[58 59 60 61] In summary, rod bipolar cells can use the differential distribution of IP_3R isoforms to produce spatio-temporally distinct cytosolic Ca^{2+} signals that contribute to Ca^{2+}-dependent functions of subcellular compartments.

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Figure Legend

FIGURE 1

Western blot showing the expression of IP_3R and RyR in ON-bipolar cells (ONBC) and controls (C). Homogenates of enzymatically isolated mouse rod bipolar cells were analyzed. Mouse cerebellum homogenate, mouse liver homogenate, mouse pancreas homogenate and mouse brain homogenate were loaded as controls for IP_3R type 1; IP_3R type 2; IP_3R type 3, and RyRs respectively. 20 μg total protein per lane were loaded on 4 – 12 % gradient gels to detect individual intracellular calcium channels. IP_3R isoform specific antibodies detected approximately 250 kDa bands, the pan-RyR antibody detected a high molecular weight band of approximately 550 kDa (numbers and arrows indicate the position and size of protein standards in kDa). Only IP_3Rs type 1 and 2 could be detected in RBCs.

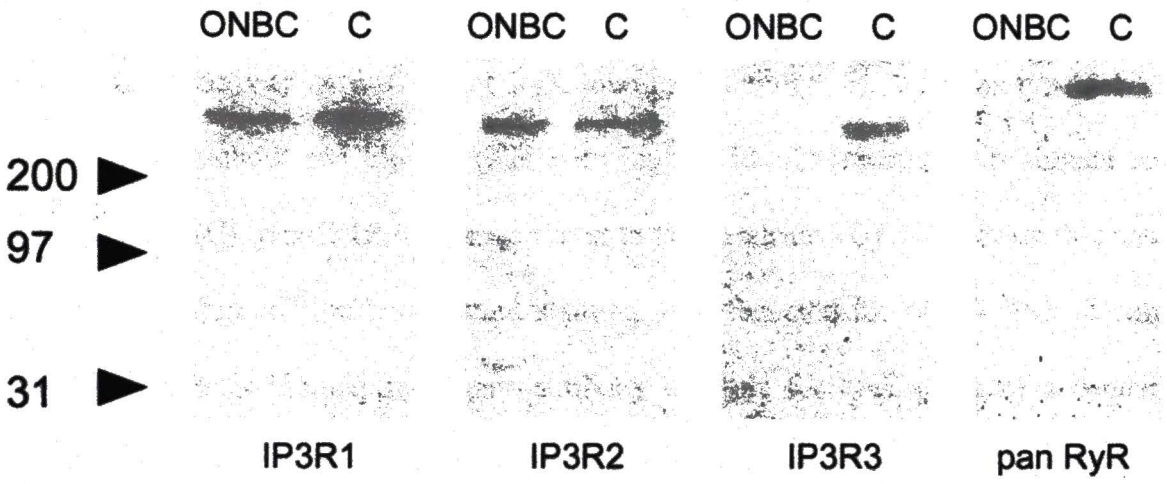

ONBC C
ONBC C
ONBC C
ONBC C
200
97
31
IP3R1
IP3R2
IP3R3
pan RyR

FIGURE 2

Localization of IP_3R 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) IP_3R immunoreactivity as well as for RyR immunoreactivity (F). Whereas type 1 IP_3R was found throughout rod bipolar cells with highest immunoreactivity in somata and axon terminals (C), type 2 IP_3R was restricted to the dendrites (D). No signals that were significantly different from background staining were detected for type 3 IP_3R (E) and RyRs (F). Controls include secondary antibody controls for both types of secondary antibodies used (A: anti-mouse; B: anti-rabbit) as well as labeling of mGluR1 and mGluR5 immunoreactivity (G and H, respectively), two upstream elements of IP_3R mediated signaling in rod bipolar cells. For each panel differential interference contrast images show the typical rod bipolar cells morphology with dendritic tree, soma axon and axon terminal system.

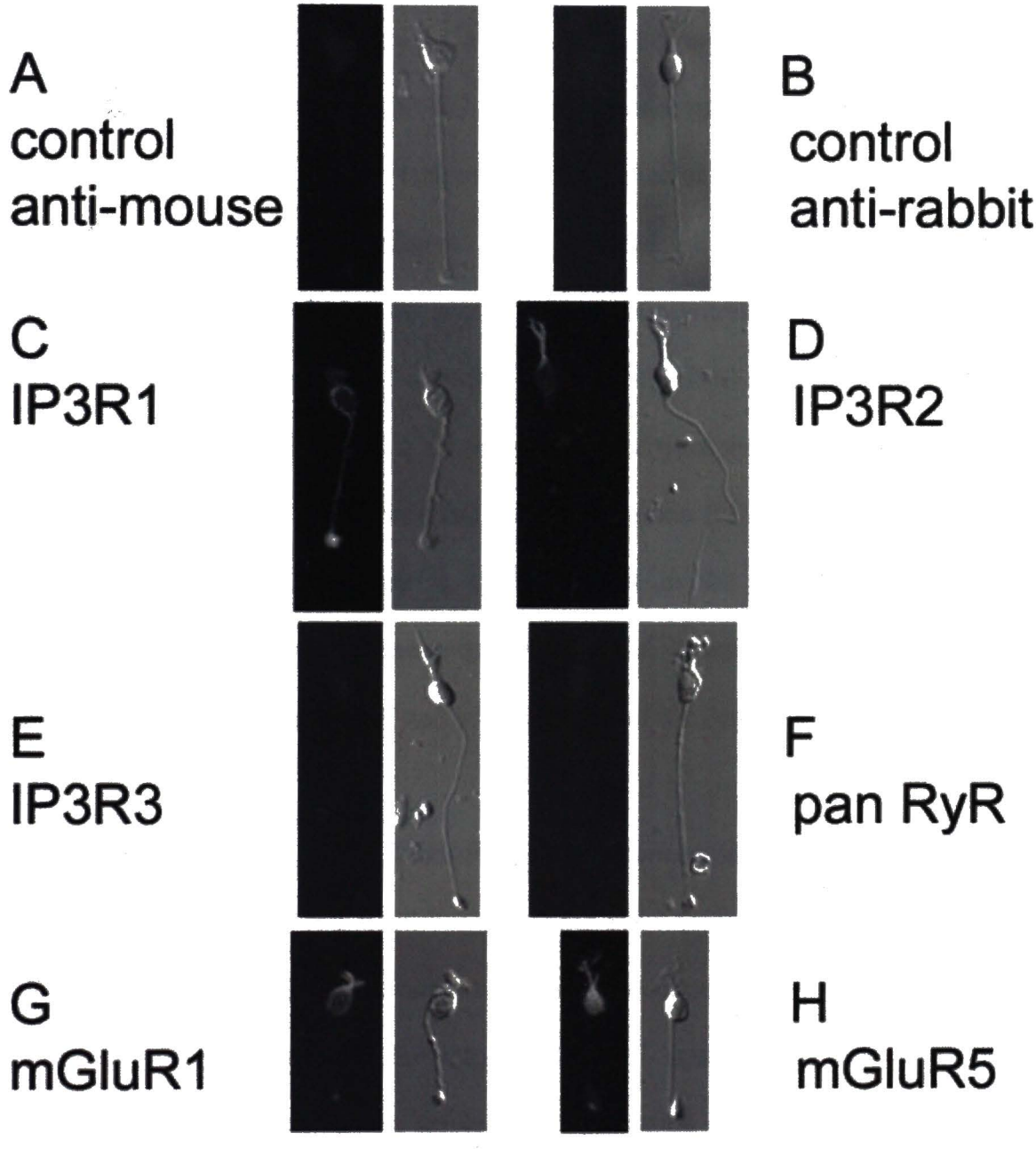
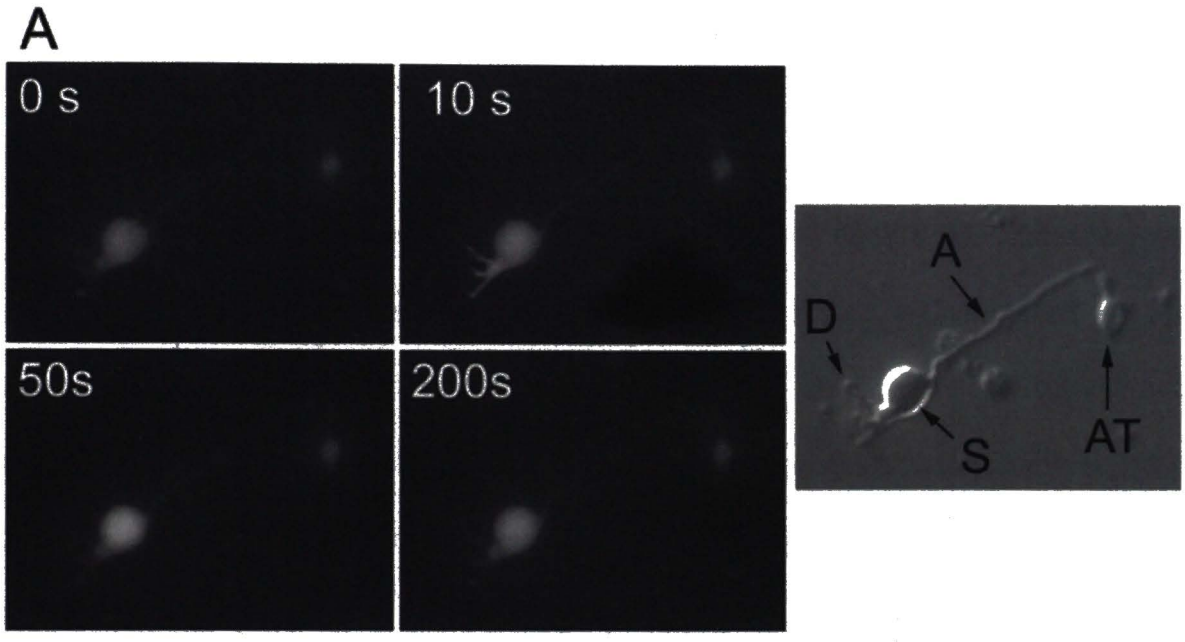

A
control
anti-mouse
B
control
anti-rabbit
C
IP3R1
D
IP3R2
E
IP3R3
F
pan RyR
G
mGluR1
H
mGluR5

FIGURE 3

Group I mGluR agonists induce spatiotemporally differential Ca^{2+} transients in isolated mouse rod bipolar cells. Intracellular Ca^{2+} concentrations were recorded optically in rod bipolar cells after ester loading with fluo-3 in the absence of extracellular Ca^{2+}. Changes in fluo-3 emission wavelength maximum as an indicator of changes in intracellular calcium concentrations were monitored. Panel A shows the montage of a representative imaging experiment visualizing the cytosolic Ca^{2+} concentration at (time point: 0 s), during (time points: 10 and 50 s), and after (time point: 200 s) application of 100 μM S-DHPG. A differential interference contrast image to the right shows the main sub-cellular regions of the rod bipolar cell, dendritic tree ("D"), soma ("S"), axon ("A"), and axon terminal system ("AT"). Whereas a Ca^{2+} transient in the dendrites was observed immediately after stimulation (time point: 10 s), a Ca^{2+} transient with larger amplitude was seen in the soma after a delay and after the peak of the dendritic Ca^{2+} transient (time point: 50 s).

Panel B shows changes in the intracellular Ca^{2+} concentration in two regions of a representative rod bipolar cell, dendrites (blue) and soma (red). Fluorescence intensity of the Ca^{2+} indicator dye fluo-3 was normalized to the baseline intensity and was used as a measure of relative changes in the intracellular Ca^{2+} concentration and displayed as intensity change over time. After a single bolus application of a low dose of an mGluR1 agonist (10 μM S-DHPG, indicated by the arrow to the left) only the dendrites responded with a Ca^{2+} transient, whereas a higher concentration of mGluR1 agonist (100 μM S-DHPG, indicated by the arrow to the right) induced Ca^{2+} transients in both dendrites and

soma. The dendritic Ca^{2+} transient followed the stimulus immediately, while the Ca^{2+} transient in the soma had a delayed onset. Ca^{2+} concentrations in both compartments returned to baseline levels during constant perfusion of the cell with medium.


A
0 s
10 s
50s
200s
A
D
S
AT

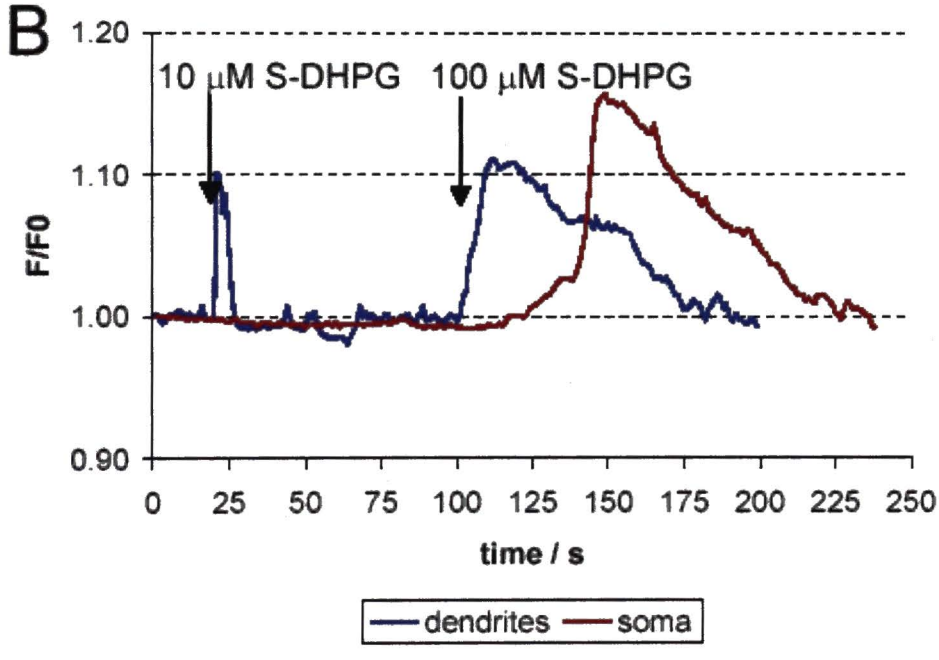
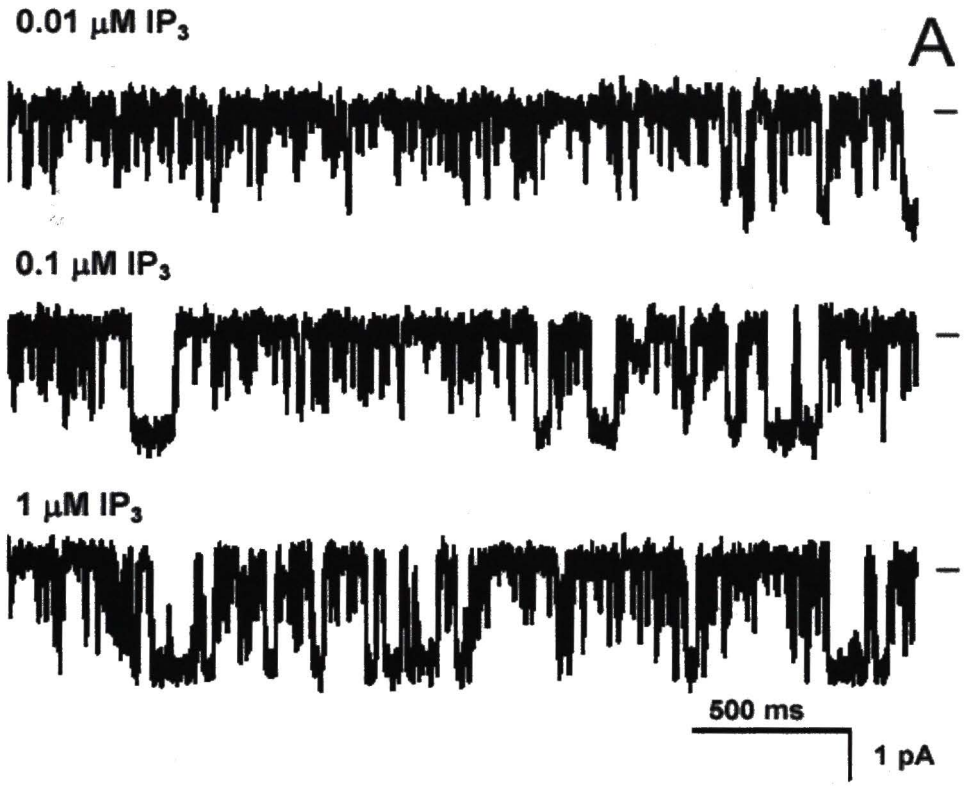
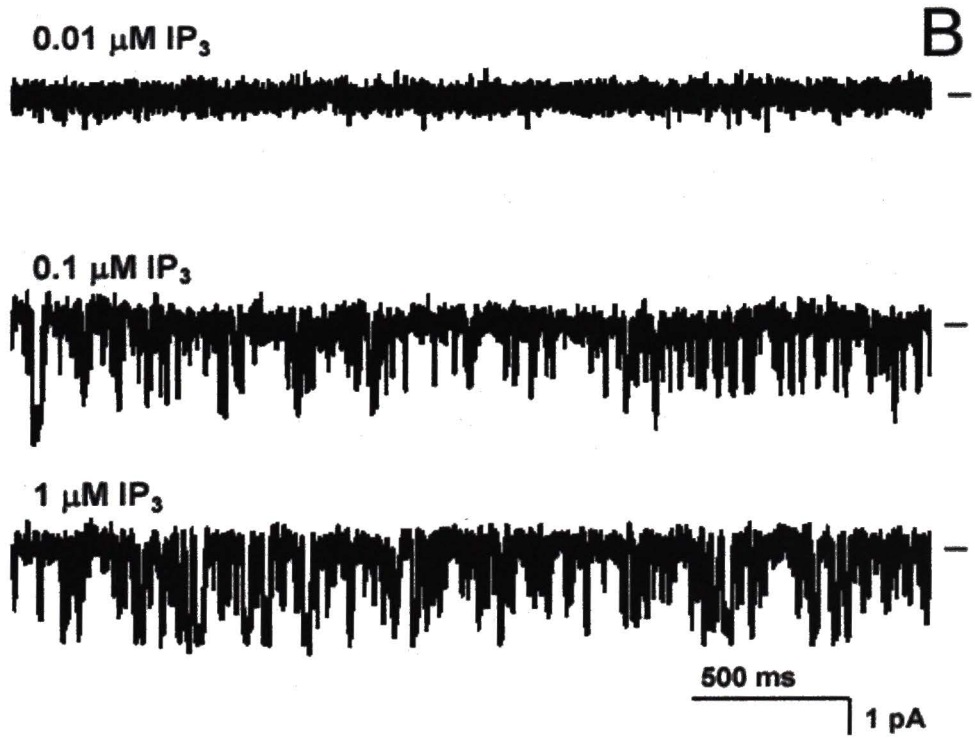
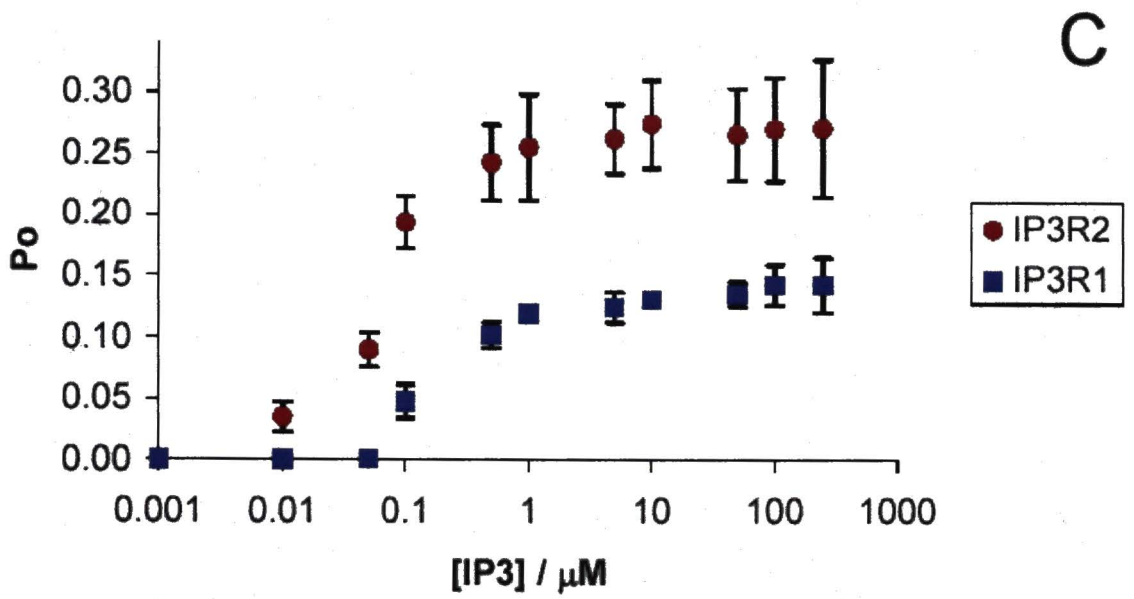

B
1.20
1.10
1.00
0.90
F/F0
10 μM S-DHPG
100 μM S-DHPG
0
25
50
75
100
125
150
175
200
225
250
time / s
dendrites
soma

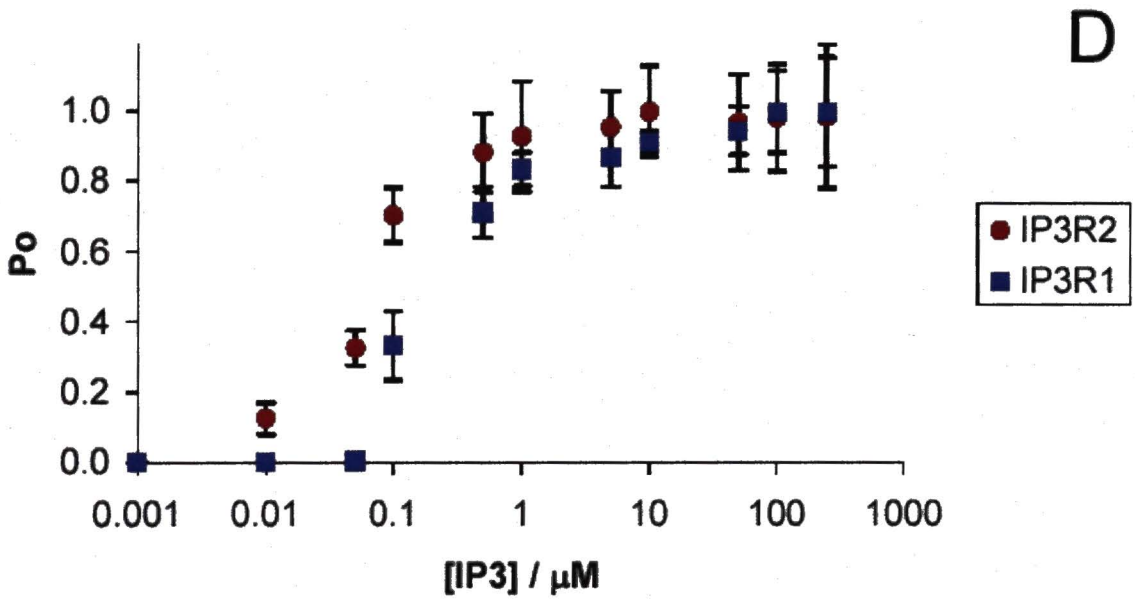
Figure 4

Two types of IP_3R with respect to their IP_3 sensitivity can be isolated from the ER of mouse rod bipolar cells. Activity of individual IP_3Rs was recorded with 0.5 μM free Ca^{2+}, 500 μM ATP as IP_3R co-agonists, 10 μM ruthenium red to block RyRs present on the cytoplasmic side of the channel and Ba^{2+} as the current carrier on the luminal side of the channel. Bars to the left of each trace indicate zero current levels of channel activity and downward deflections indicate channel openings. A range of physiologically relevant concentrations of IP_3 on the cytoplasmic side of the channel was tested to evaluate activation of the channels by their ligand. Panels A and B of figure 4 show electrophysiological recordings of two representative experiments with three traces at 0.01, 0.1 and 1 μM IP_3 exemplifying the two types of IP_3Rs that could be observed in mouse rod bipolar cells. Whereas one type of IP_3R showed activation by low IP_3 concentrations (A), the second type was activated by intermediate levels of IP_3. Panels C and D summarize the IP_3 dependence of the absolute and normalized open probability respectively for the two types of IP_3Rs isolated from mouse rod bipolar cells. The IP_3R with high IP_3 sensitivity is identified as IP_3R 2 (type 2 IP_3R; n=9) and the IP_3R with intermediate IP3 sensitivity as IP_3R 1 (type 1 IP_3R; n=11).


0.01 μM IP3
A
0.1 μM IP3
1 μM IP3
500 ms
1 pA


0.01 μM IP3
B
0.1 μM IP3
1 μM IP3
500 ms
1 pA


C
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Po
0.001
0.01
0.1
1
10
100
1000
[IP3] / μM
IP3R2
IP3R1


D
1.0
0.8
0.6
0.4
0.2
0.0
Po
0.001
0.01
0.1
1
10
100
1000
[IP3] / μM
IP3R2
IP3R1

CHAPTER IV

REGULATION OF CYTOSOLIC CALCIUM LEVELS BY RYR INTRACELLULAR CALCIUM CHANNELS IN ROD BIPOLAR CELLS

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Running Title: Ca^{2+} signaling in rod bipolar cells

Key words: ion channel, neuron, immunochemistry, calcium release, intracellular calcium stores, calcium imaging

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REGULATION OF CYTOSOLIC CALCIUM LEVELS BY RYR INTRACELLULAR CALCIUM CHANNELS IN ROD BIPOLAR CELLS

ABSTRACT

PURPOSE. The present study analyzed the localization of the different isoforms of ryanodine receptors (RyRs) and determined their functional influence on cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) in mouse rod bipolar cells. We hypothesize that rod bipolar cells differentially express distinct isoforms of RyRs. RyR are intracellular Ca^{2+} release channels that contribute to neuronal Ca^{2+} signaling.

METHODS. Murine adult retinal neurons were isolated by enzymatic dissociation. Immunofluorescence studies were performed to determine the expression and localization of RyRs in rod bipolar cells. The cytosolic Ca^{2+} concentration of acutely isolated rod bipolar cells was monitored optically with microspectrofluorimetry and the function of intracellular Ca^{2+} release channels was measured with pharmacological stimulation.

RESULTS. Immunocytochemical analyses of RyR localization demonstrated that mouse rod bipolar cells express isoforms of RyRs with distinct subcellular distribution patterns. Increases in cytosolic Ca^{2+} were measured after RyRs were activated with the agonist caffeine. Ca^{2+} transients were decreased by pretreatment of cells with the RyR antagonist dantrolene. Depolarization of rod bipolar cells with 50mM KCl in the extracellular medium resulted in a robust increase in intracellular Ca^{2+} which was reduced, but not abolished in the presence of the RyR antagonist dantrolene, indicating these intracellular

Ca^{2+} channels contribute to depolarization induced cytosolic Ca^{2+} transients in rod bipolar cells.

CONCLUSIONS. RyR Ca^{2+} channels are involved in Ca^{2+} signaling in rod bipolar cells and represent potentially function as pharmacological targets in diseases of the neural retina involving Ca^{2+} dyshomeostasis.

Introduction

Neuronal cells increase cytosolic Ca^{2+} concentration $[Ca^{2+}]_c$ through two signaling pathways, activation of Ca^{2+} channels on the plasma membrane and Ca^{2+} release from the intracellular stores. The endoplasmic reticulum (ER) as an intracellular Ca^{2+} store release Ca^{2+} primarily via activation of inositol 1,4,5-trisphosphate receptors (IP_3R) and ryanodine receptors (RyR) (Marshall and Taylor, 1993; Carafoli et al., 2001; Fill and Copello, 2002; Banerjee and Hasan, 2005).

To date, three types of RyRs have been shown to exist. RyR types 1, 2, and 3 are encoded by three distinct genes and are found in a variety of mammalian tissues typically displaying differential distribution patterns, including cardiac tissue (Lai et al., 1992; Meissner, 1994; Otsu et al., 1990), skeletal muscle (Coronado et al., 1994; Meissner, 1994; Shoshan-Barmatz and Ashley, 1998; Sutko and Airey, 1996), brain (Lai et al., 1992; McPherson and Campbell, 1990), and smooth muscle (Herrmann-Frank et al., 1991). The different types of RyR share 65-70% sequence homology at the amino acid sequence level (Coronado et al., 1994; Meissner, 1994; Shoshan-Barmatz and Ashley, 1998).

Within the vertebrate retina, regulation of the transduction process and the visual signal cascade involves modulation of $[Ca^{2+}]_c$ (Krizaj et al., 1999; Fain et al., 2001; Akopian and Witkovsky, 2002; Hurtado et al., 2002; Krizaj and Copenhagen, 2002; Chavez et al., 2006; Suryanarayanan and Slaughter, 2006), and RyR function has been implicated in these processes. Ryanodine-sensitive stores play a dynamic role in controlling presynaptic $[Ca^{2+}]_c$ in sensory neurons such as in inner hair cells (Kennedy and Meech, 2002).

Within the retina, bipolar cells are the first inter-neurons within the vertical pathway's glutamatergic system, where ON-bipolar cells comprise both ON-cone and rod bipolar cells (Ghosh et al., 2004; Euler and Wässle, 1995). Bipolar cells are important for the processing of retinal information and the integration of signals between the two synaptic layers of the retina (Brandstätter et al., 1998; Koulen, 1999). In the invertebrate retina, RyRs contribute to light adaptation processes (Akopian and Witkovsky, 2002; Akopian et al., 1998; Arnon et al., 1997; Baumann, 2000). Immunolocalization studies have also shown that RyRs are expressed within several retinal neuron cell types in non-mammalian vertebrates (Akopian et al., 1998; Krizaj et al., 2003; Krizaj et al., 2004). Despite the evidence in these studies, expression and localization of RyR types 1, 2, and 3 in the mammalian retina have yet to be fully addressed.

In the present study, we analyzed the localization of the different RyRs types and determined their influence on $[Ca^{2+}]_c$ in mouse rod bipolar cells.

Material 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 of rod bipolar cells

Rod bipolar cells were isolated by mechanical and enzymatic dissociation of retina. Retinas from adult outbred Swiss Webster mice were dissected and incubated in L-15 solution containing 1g/L pronase and 2 mM EGTA for 20 minutes in a water bath at 37°C. The tissue was washed with L-15 containing 0.5% bovine serum albumin (BSA), 2 mM EGTA, and 10 mg/L DNase, and the cells were obtained by trituration of the tissue. Isolated cells were plated on poly-L-lysine–coated coverslips and incubated in L-15 at 37°C for 30 minutes prior to experiments. Only rod bipolar cells which maintained their intact morphology and their physiological properties, i.e. the capacity for depolarization and regulation of changes in $[Ca^{2+}]_c$, were used in experiments.

Immunocytochemistry

Immunocytochemistry was performed as previously described (Koulen et al., 1999; Koulen et al., 2005). Briefly, cells were fixed to coverslips using 4% para-formaldehyde (w/v) in phosphate buffer and incubated in blocking solutions for 1 hour. Over-night incubation in primary custom RyR type 1, 2, and 3 antibody (1:500,

Chemicon, AB72886, AB82896, and AB92888 respectively) was followed by washing and incubation with secondary antibody solutions for 1 hour. Goat anti-Rabbit IgG labeled with Alexa 488 (diluted 1:1000, Molecular Probes, Carlsbad, CA) was used as a secondary antibody to visualize immunoreactivity via standard immunofluorescence microscopic techniques. Coverslips were washed to remove excess antibody, after which they were mounted using Prolong Gold anti-fade reagent with DAPI (Molecular Probes, Carlsbad, CA), to visualize the nuclei.

Immunohistochemistry

Immunohistochemistry was carried out as previously described (Koulen and Brandstätter, 2002; Kaja et al., 2002). Briefly, mice were anesthetized deeply with CO_2, and euthanized by decapitation. The eyes were removed and retinae were dissected. The tissue was immersion fixed in 4% (w/v) paraformaldehyde (PA) in phosphate buffer (PB; 0.1 M, pH 7.4) for 30 minutes, cryoprotected in 10%, 20%, and 30% sucrose (w/v) in PBS at 4°C. Retinae were frozen in OCT, cryo-sectioned at 12 µm thickness, and collected on slides. Immunoreactivity was detected as described above for immunocytochemistry.

Imaging and quantification of $[Ca^{2+}]_c$ transients

Ca^{2+} imaging was performed as described previously (Koulen et al., 1999; Koulen et al., 2005; Westhoff et al., 2003; Hwang et al., 2003). Briefly, acutely isolated rod bipolar cells were loaded with 4 µM fluo-3-AM (fluo-3-acetoxymethylester; Molecular

Probes, Eugene, OR) at 37°C for 30 min, washed, and recorded for up to six hours after dissociation. The changes in fluorescence intensity of the Ca^{2+} indicator fluo-3 in loaded cells was 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. 6.5, Compix Inc., Imaging Systems / Hamamatsu Photonics Management Corporation, Bridgewater, NJ). Images were acquired every 0.5 second, and Ca^{2+} transients were calculated as the ratio of the fluorescence intensity during drug application (F) over the average baseline fluorescence intensity 10s before drug application (F/F0). The soma and axon terminal of rod bipolar cells were analyzed independently, and their spatiotemporal Ca^{2+} transient patterns measured individually.

During the Ca^{2+} imaging experiments, the ryanodine receptor agonist caffeine (Sigma-Aldrich, St. Louis, MO) was applied to the imaging chamber at pharmacologically relevant concentrations. In several experiments, pretreatment for 30 minutes with 100 μM dantrolene (Sigma-Aldrich) was performed to fully block RyR activity (Koulen and Thrower, 2001). The dependence of RyR-mediated Ca^{2+} transients on extracellular Ca^{2+} concentration was measured by replacing the extracellular medium with Ca^{2+}-free L-15 medium containing 5 mM EGTA. When $[Ca^{2+}]_c$ were recorded in Ca^{2+}-free medium, cells were equilibrated in Ca^{2+}-free medium for two minutes prior to the application of RyR agonists. No significant depletion of Ca^{2+} stores occurred during this time. To test the involvement of RyR-dependent Ca^{2+} transients during

depolarization of rod bipolar cells, cells were pretreated with 100 μM dantrolene for 30 minutes prior to chemical depolarization in L-15 medium containing 50 mM KCl.

Results

In the present study, the distribution of RyRs in rod bipolar cells of the mouse retina was determined and function of RyR activation in intracellular Ca^{2+} signaling was measured. To visualize the expression of the different RyR isoforms in the retina, vertical cryosections of the mouse retina were prepared and immunostained. RyR type 3 immunoreactivity was detected in the retinal pigment epithelium (RPE), regions associated with bipolar cells, the outer plexiform layer (OPL), inner nuclear layer (INL), the inner plexiform layer (IPL), and in the ganglion cell layer (GCL). RyR type 1 immunoreactivity was found in the GCL and the nerve fiber layer (NFL). RyR type 2 displayed immunoreactivity within the regions associated with bipolar cell expression, the OPL, INL, and the IPL (Fig. 1).

The distribution of RyR immunoreactivity was further corroborated using specific antibodies and immunofluorescence cytochemical stainings of acutely isolated mouse rod bipolar cells (Fig. 2). Immunoreactivity for RyR type 3 was detected throughout the cell while immunoreactivity for RyR type 2 was detected primarily in the dendrites and cell body and no immunoreactivity for RyR type 1 above back ground control levels was measured. The differential expression of RyR types within rod bipolar cells indicate that Ca^{2+} signaling patterns within the axon terminal are likely RyR type 3 mediated, while both RyR type 2 and 3 could contribute to Ca^{2+} signaling in the outer parts of the cell.

The staining patterns in isolated RBC corroborate the immunohistochemistry data of differentially distributed RyR immunoreactivities.

To correlate the localization data with the function of RyRs, Ca^{2+} transients of isolated rod bipolar cells were measured in response to pharmacological activation of RyRs. Rod bipolar cells were stimulated with bolus applications of caffeine, an agonist of RyRs, which induced Ca^{2+} transients within the distal portion of rod bipolar cells (Fig. 3). Figure 3 shows an image montage of a representative rod bipolar cell's response to caffeine stimulation and the spatiotemporal pattern of the associated Ca^{2+} transient. A differential interference contrast image (DIC) shows the main subcellular regions of the rod bipolar cell, dendritic tree (D), soma (S), axon (A), and axon terminal system (AT).

Recordings of the Ca^{2+} transients were measured after application of 10 mM caffeine in Ca^{2+} containing and Ca^{2+} free L-15 medium. Treatment with vehicle alone elicited no significant Ca^{2+} response compared to baseline levels (Fig. 4A). Application of caffeine resulted in an increase in $[Ca^{2+}]_i$ measured as fluo-3 emission fluorescence intensity (Fig. 4B). Ca^{2+} responses measured in medium with Ca^{2+} and in Ca^{2+}-free medium were both significantly greater in amplitude than that of vehicle controls (data not shown) with Ca^{2+} transients recorded in Ca^{2+}-containing medium producing the largest increases in $[Ca^{2+}]_i$. When extracellular Ca^{2+} was absent, distinct responses of Ca^{2+} release from intracellular stores were measured. To further identify that the Ca^{2+} transients were generated by RyRs, cells were pretreated with 100μM dantrolene (RyR antagonist) prior to agonist application. Pretreatment with dantrolene abolished the agonist induced Ca^{2+} response (Fig. 4C) indicating that pharmacological blockage of

RyRs prevents the caffeine-induced Ca^{2+} transients and a contribution of RyRs to RBC Ca^{2+} signaling.

Subsequently the role of RyRs during chemically induced depolarization in rod bipolar cells was measured. Application of 50 mM KCl in the extracellular medium led to an increase in $[Ca^{2+}]_i$ (Fig. 5B) that was significantly greater than that of vehicle controls (Fig. 5A). The depolarization mediated increase in $[Ca^{2+}]_i$ was greatly reduced when neurons were pretreated with 100μM dantrolene (Fig. 5C). Rod bipolar cells treated with caffeine in L-15 medium had an AUC and maximal amplitude that was significantly higher than experiments performed in Ca^{2+}-free medium and the vehicle treated control. Maximal amplitude was also significantly higher when comparing caffeine stimulation in Ca^{2+}-free medium and the vehicle treated control (Fig. 6). Rod bipolar cells that were chemically depolarized had an AUC and maximal amplitude that was significantly higher than experiments where cells were pretreated with antagonist and the vehicle treated control. Cell pretreated with xestospongin D also had significantly higher AUC and maximal amplitudes than that of the dantrolene pretreated cells and vehicle treated control (Fig. 7).

Analysis of kinetic parameters to determine the maximal amplitude and AUC were also performed to provide insight into the amount of Ca^{2+} being released from these rod bipolar cells and the statistical significance of these changes (Table 1).

Discussion

The present study provides evidence that RyR-induced Ca^{2+} responses in rod bipolar cells are mediated by RyR type 2 and 3, where type 3 is involved with Ca^{2+} release form the axon terminal. Mechanical and enzymatic dissociation of freshly isolated rod bipolar cells does not alter their substructural morphology, and more importantly their physiological functional properties remain the same (Vaquero et al., 1996; Klumpp et al., 1995; Wässle et al., 1991; Greferath et al., 1995; Enz et al., 1996; Feigenspan et al., 1993). Therefore, they serve as a good model for RyR functional studies.

Bipolar cells are also the first interneurons in the glutamatergic vertical pathway that integrate visual information between the two synaptic layers of the retina (Brandstatter et al., 1998; Koulen, 1999). Being the second order neurons within the vertical pathway, it has been shown that modulation of intracellular Ca^{2+} concentration is important for neurotransmitter release (Maple et al., 1994; Matsui et al., 1998; Matthews, 1999; Gomis et al., 1999; Tachibana, 1999; Neves et al., 2001; Burrone et al., 2002; Singer and Diamond, 2003). Rod bipolar cells express the group I metabotropic glutamate receptor, mGluR1 and mGluR5 (Koulen et al., 1997; Koulen et al., 2005) that are coupled to IP_3R-mediated Ca^{2+} release (Peng et al., 1991; Day et al., 1993; Micci and Christensen, 1996; Lopez-Colome and Lee, 1996). mGluR1/5 activation with its agonist (S)-3,5-dihydroxyphenylglycine (S-DHPG) initiates Ca^{2+} transients that are agonist-concentration dependent, i.e. dependent on the strength of the glutamate input (Koulen et al., 2005).

Differential distribution of IP_3Rs in rod bipolar cells had been identified previously (Koulen et al., 2005). This distribution of IP_3Rs allows distinct Ca^{2+} signals to be generated within a particular subcellular compartment of RBC (Koulen et al., 2005). This paradigm can be used by neurons to produce a multiplicity of Ca^{2+} signals and regulate the strength of a Ca^{2+} signaling in different tissue. RyRs are Ca^{2+}-induced Ca^{2+} release channels that can be activated via Ca^{2+} influx from voltage-gated Ca^{2+} channels or receptor-operated Ca^{2+} channels, triggering Ca^{2+} release from SR/ER (Kuba, 1994). The infux of Ca^{2+} from intracellular stores elicited by IP_3R could be further potentiated by activation of the RyR within rod bipolar cells, leading to different spatial and temporal signaling. Therefore, they have the capacity to contribute to the diversity of signals and signal strength in rod bipolar cells resulting in the spread of highly localized Ca^{2+} signals to neighboring subcellular areas and potentially generating global Ca^{2+} waves (Berridge et al., 1998).

RyR regulation of $[Ca^{2+}]_c$ in RBC potentially contributes to the light adaptation process as well as in the regulation of Ca^{2+} homeostasis. Upon release of glutamate by photoreceptor cells glutamate onto ON-bipolar cells results in hyperpolarization via activation of mGluR6, inactivating cGMP-gated cation channels on the plasma membrane and resulting in a sign inversion (Nakanishi et al., 1994). Modulation of cytosolic Ca^{2+} controls the activity of cGMP-gated Ca^{2+} channels in RBCs, contributing to adaptation of the ON-pathway and changing light levels during visual neurotransmission (Shiells and Falk 1999, Nawy 2000, and Nawy 2004), reviewed in (Shiells, 1999).

Acknowledgements

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Berridge, M.J., Lipp, P. & Bootman, M.D. (2000) The versatility and universality of calcium signalling. Nat. Rev. Mol. Cell Biol., 1, 11–21.

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Figure Legend

FIGURE 1

Immunohistochemical of vertical cryostat sections identifying the RyR immunoreactivity in the adult mouse retina. **D:** Negative control with primary antibody omitted. **A:** RyR type 1. **B:** RyR type 2. **C:** RyR type 3. Red immunofluorescence identifies the respective RyR immunoreactivity, nuclei are counterstained with DAPI and shown in blue, and the retinal layers are visualized with differential interference contrast (DIC) imaging. RyR type 1 immunoreactivity was found found in the ganglion cell layer (GCL) and the nerve fiber layer (NFL). RyR type 2 displayed immunoreactivity in the retinal pigment epithelium (RPE) and within the regions associated with bipolar cell expression, the outer plexiform layer (OPL), inner nuclear layer (INL), and the inner plexiform layer (IPL). RyR type 3 immunoreactivity was detected in the RPE, regions associated with bipolar cells, the OPL, the INL, the IPL, and in the GCL. This corresponds with immunocytochemistry data of the rod bipolar cells.

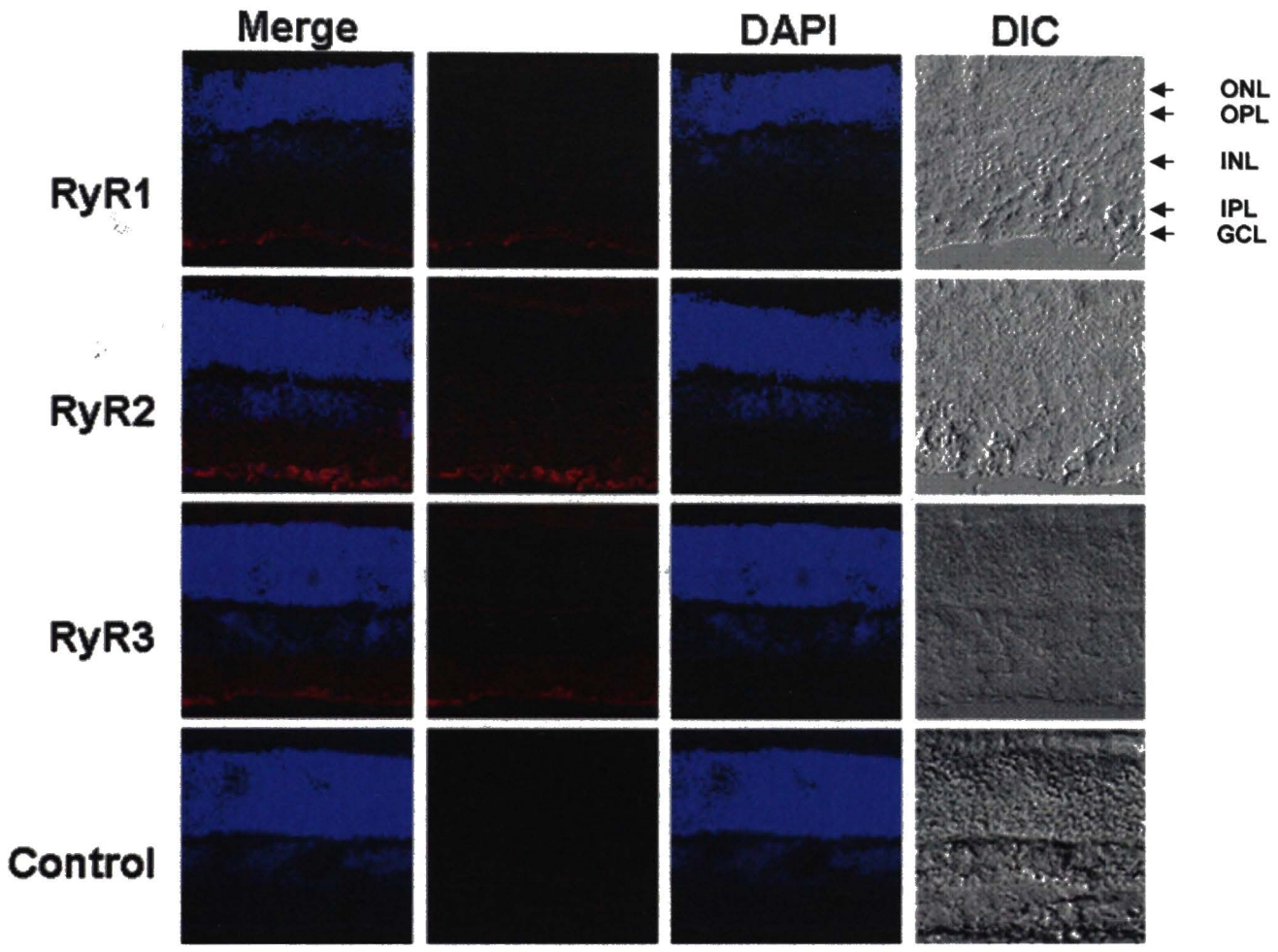

Merge
DAPI
DIC
RyR1
RyR2
RyR3
Control
ONL
OPL
INL
IPL
GCL

FIGURE 2

Localization of RyR immunoreactivity in isolated rod bipolar cells of the adult mouse retina. Acutely isolated rod bipolar cells were fixed and stained for type 1 (**B**), 2 (**C**), type 3 (**D**), RyR immunoreactivity. **(A)** Controls consisted in the omission of primary antibodies. For each panel, differential interference contrast images show the typical rod bipolar cell morphology with dendritic tree, soma axon, and axon terminal system. No immunoreactivity for RyR type 1 above back ground control levels was measured. RyR type 2 displayed immunoreactivity within the dendrites and soma of rod bipolar cells. Immunoreactivity for RyR type 3 was detected throughout the cell.

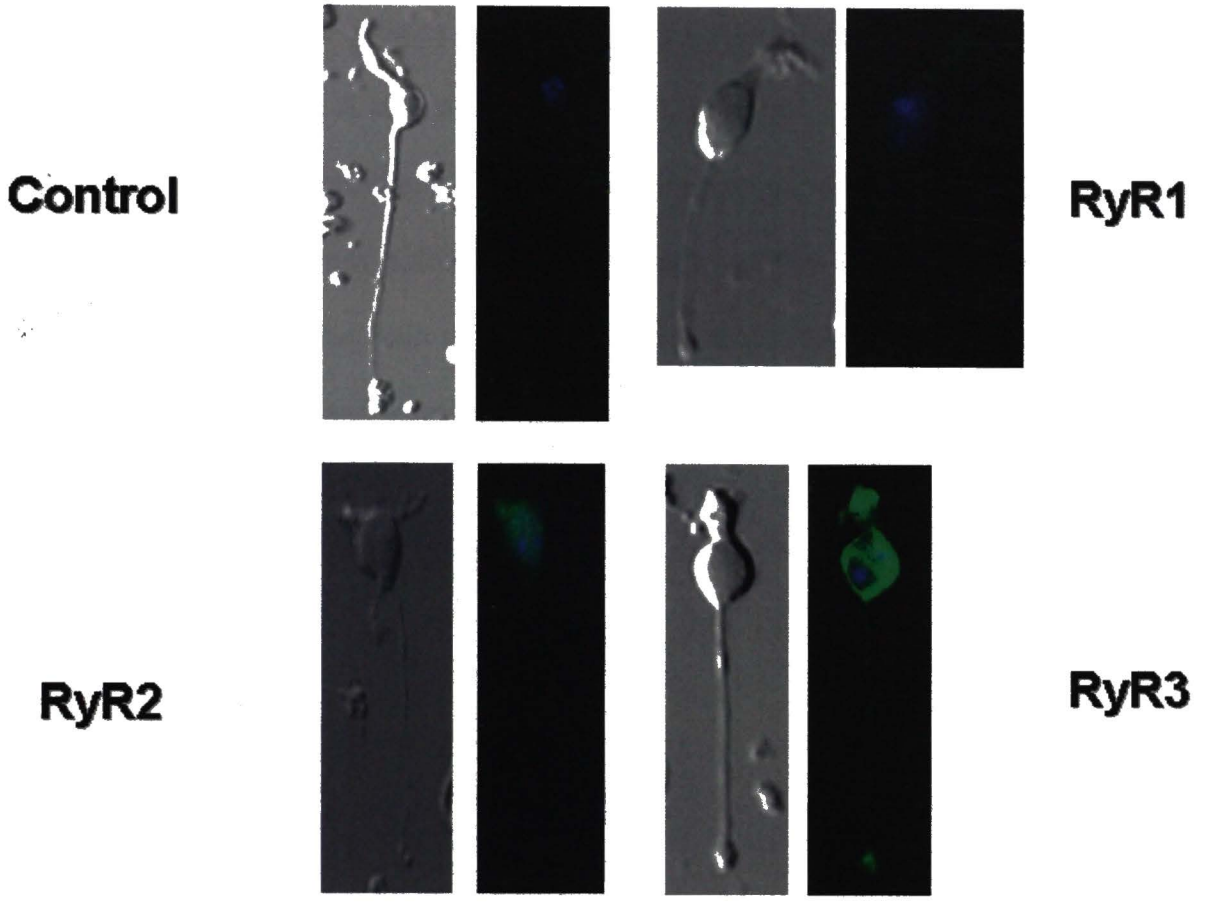

Control
RyR1
RyR2
RyR3

FIGURE 3

The RyR agonist caffeine induces spatiotemporally differential Ca^{2+} transients in isolated mouse rod bipolar cells. A montage of a representative imaging experiment visualizing the cytosolic Ca^{2+} concentration at 10 seconds, before application, 100, and 200 seconds, after application of 10 mM caffeine was observed. A differential interference contrast image to the left shows the main subcellular regions of the rod bipolar cell, dendritic tree (D), soma (S), axon (A), and axon terminal system (AT). Scale bar =10 μm.

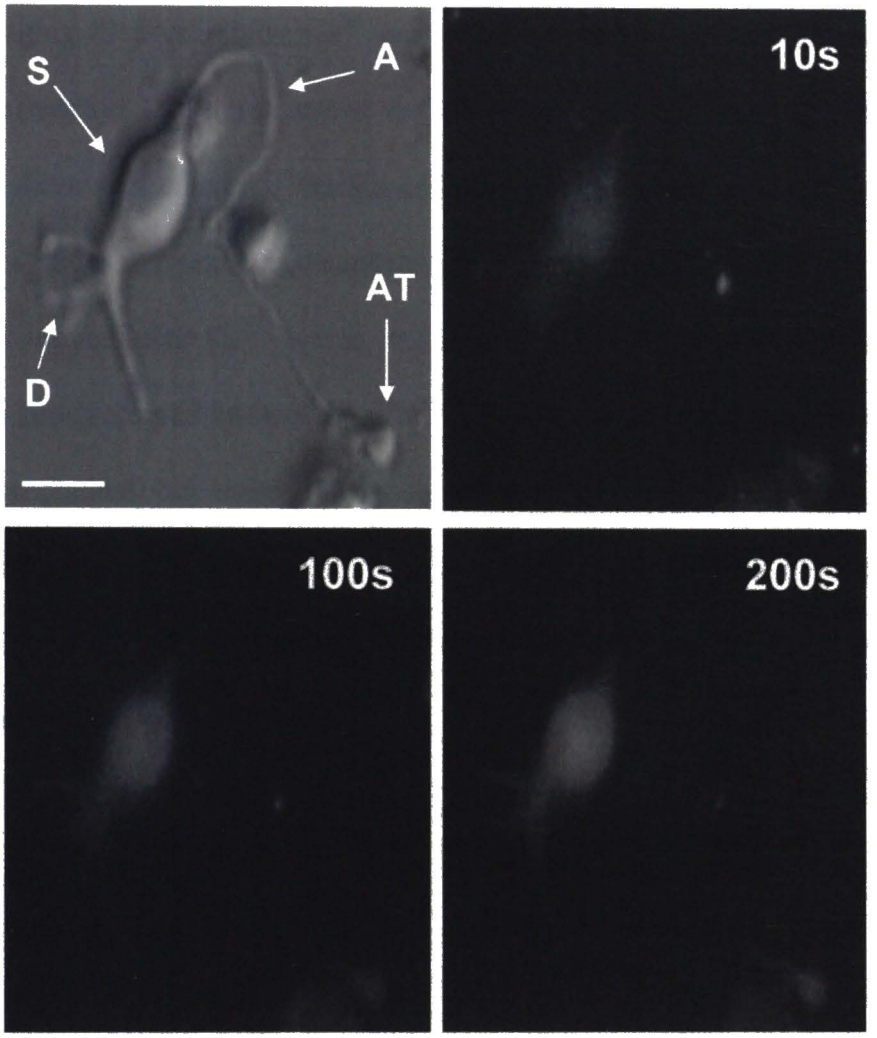

S
A
AT
D
10s
100s
200s

FIGURE 4

Optical imaging of intracellular Ca^{2+} concentrations in caffeine-stimulated rod bipolar cells. Representative Ca^{2+} transients in the axon terminal and somata of rod bipolar cells are shown. Acutely isolated rod bipolar cells were ester-loaded with fluo-3-AM dye and exposed to vehicle in control experiments, **A.** Vehicle control treatment did not lead to changes in intracellular Ca^{2+} levels. **B.** Addition of 10mM caffeine, initiated Ca^{2+} oscillations and increased intracellular Ca^{2+} levels in rod bipolar cell axon terminals, but generated a gradual but steady increase within the soma. These intracellular Ca^{2+} transients were abolished when RyRs were pre-treated with the RyR blocker, dantrolene, **C**. Intracellular Ca^{2+} release was measured as the ratio of fluorescence over the average initial baseline fluorescence (F/F0).

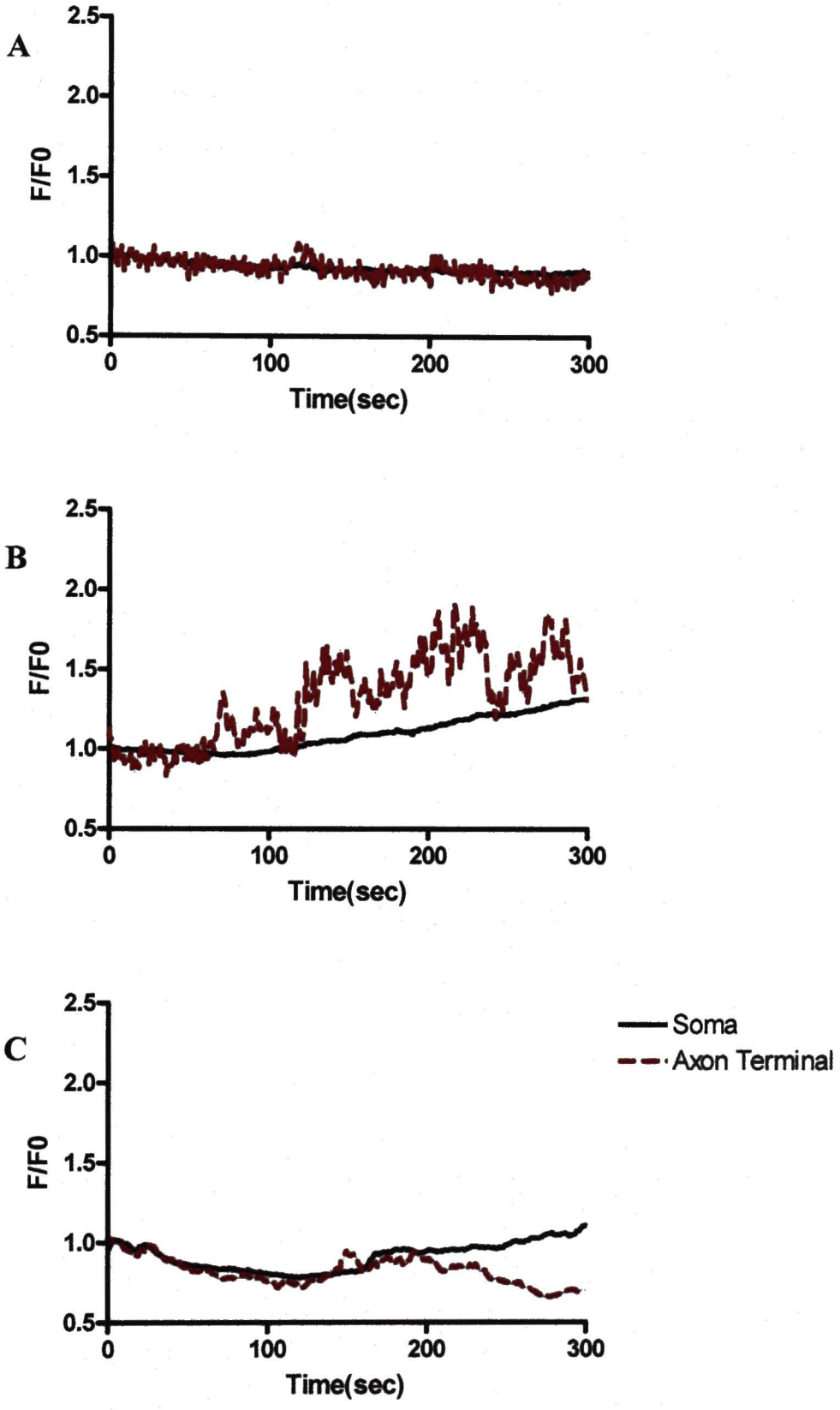

A
B
C
F/F0
Time(sec)
2.5
2.0
1.5
1.0
0.5
0
100
200
300
Soma
Axon Terminal

FIGURE 5

Optical imaging of intracellular Ca^{2+} during chemical depolarization of rod bipolar cells. Acutely isolated rod bipolar cells were ester-loaded with fluo-3-AM dye and emission fluorescence was monitored. **A.** No changes in intracellular Ca^{2+} levels were detected after application of vehicle in control experiments. **B.** Exposure to medium containing 50mM KCl initiated an increase intracellular Ca^{2+} levels in rod bipolar cell. **C.** The increase in intracellular Ca^{2+} was abolished when cells had been incubated with the RyR blocker, dantrolene.

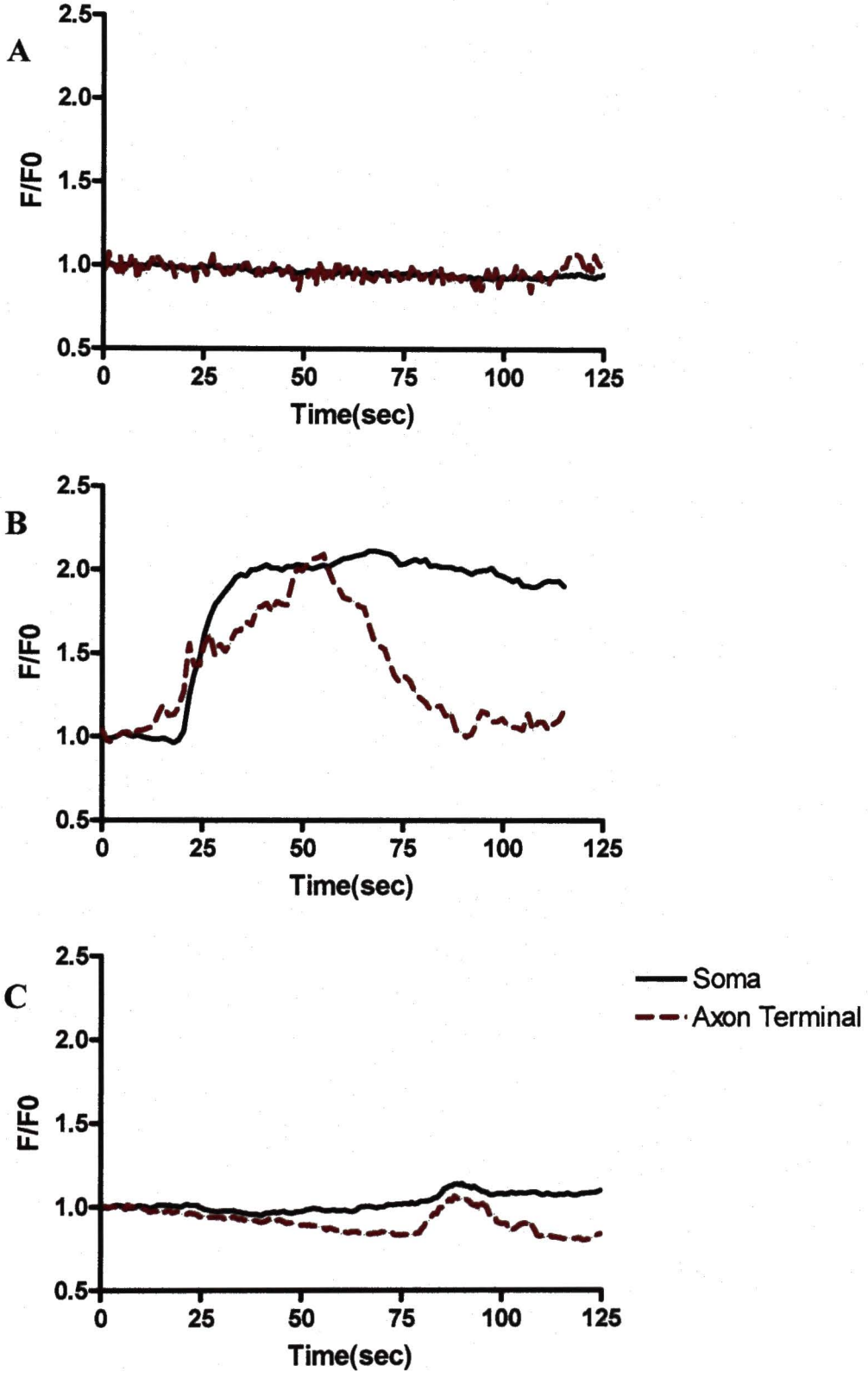
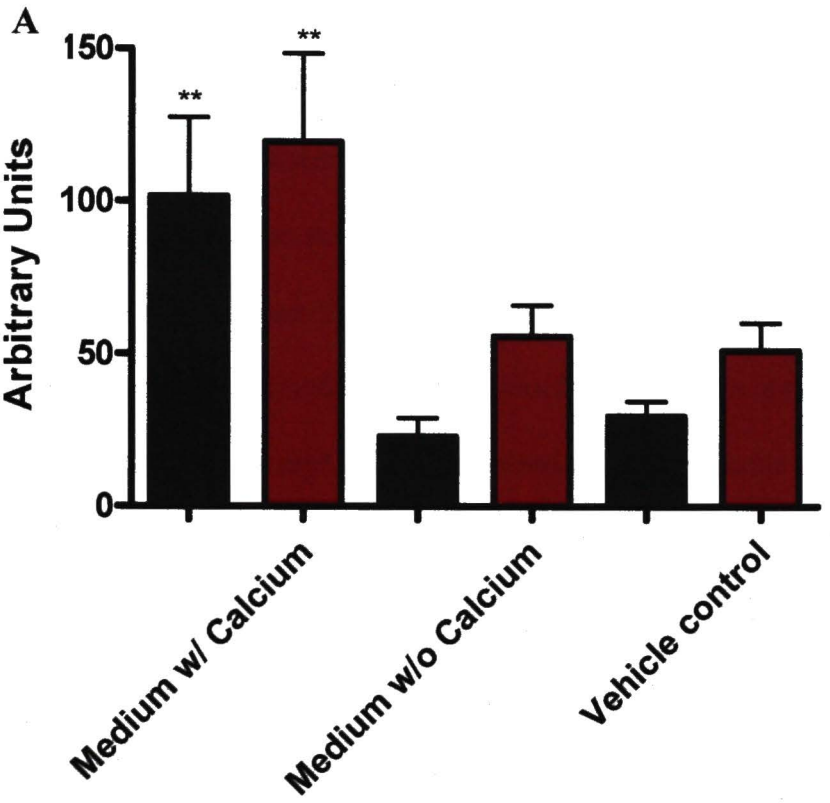

A
B
C
F/F0
Time(sec)
2.5
2.0
1.5
1.0
0.5
0
25
50
75
100
125
Soma
Axon Terminal

FIGURE 6

Kinetic parameters of pharmacological modulation in Rod Bipolar Cells. Rod bipolar cells were ester-loaded with fluo-3, treated with either vehicle (L-15 media) or caffeine. In separate experiments cells were pretreated with dantrolene to identify the RyR mediated component of Ca^{2+} transients. Parameters of Ca^{2+} transients were determined in the soma and axon terminal. **A.** The area under the curve (AUC) of caffeine-induced Ca^{2+} transients in rod bipolar cells was calculated. **B.** The maximal amplitude of caffeine-induced Ca^{2+} transients was measured. Rod bipolar cells treated with caffeine in L-15 medium had an AUC and maximal amplitude that was significantly higher than experiments performed in Ca^{2+}-free medium and the vehicle treated control. Maximal amplitude was also significantly higher when comparing caffeine stimulation in Ca^{2+}-free medium and the vehicle treated control.


A
Arbitrary Units
150
100
50
0
**
**
Medium w/ Calcium
Medium w/o Calcium
Vehicle control

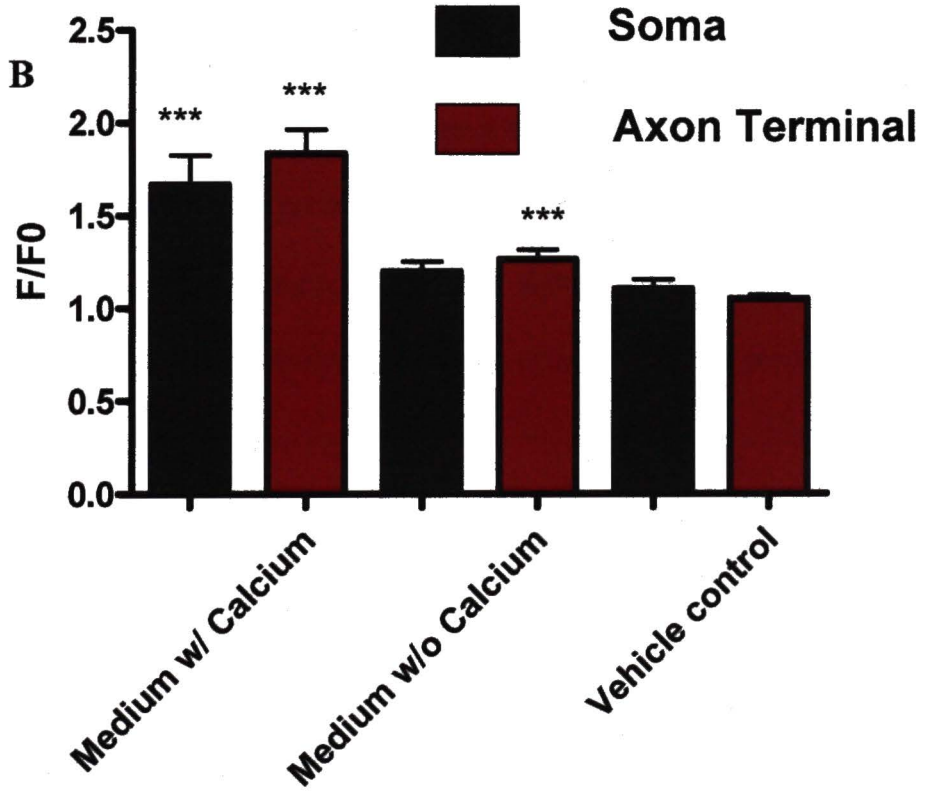
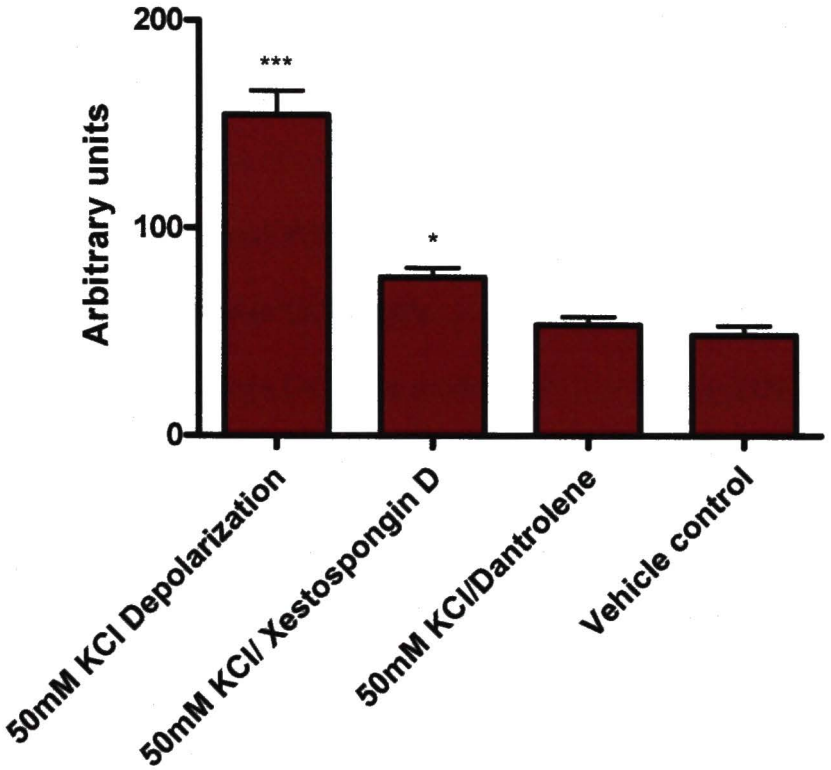

B
F/F0
2.5
2.0
1.5
1.0
0.5
0.0
***
***
***
Soma
Axon Terminal
Medium w/ Calcium
Medium w/o Calcium
Vehicle control

FIGURE 7.

Kinetic parameters of depolarization in Rod Bipolar Cells. Rod bipolar cells were again ester-loaded with fluo-3, and chemically depolarized in separate experiments where cells were pretreated with dantrolene and xestospongin D to determine the contribution of the store operated channels to intracellular Ca^{2+} transients during depolarization. Parameters of Ca^{2+} transients were determined in the axon terminal. The area under the curve (AUC) of the Ca^{2+} transients from 50 mM KCl depolarized rod bipolar cells was calculated. The AUC of the 50 mM KCl -mediated transients was measured vs rod bipolar cells that were also depolarized but pretreated with the RyR antagonist dantrolene and the IP_3R antagonist xestospongin D, and vehicle-mediated transients are shown for the rod bipolar cell axon terminal. **A.** The area under the curve (AUC) of depolarized-induced Ca^{2+} transients in rod bipolar cells was calculated. **B.** The maximal amplitude of depolarized-induced Ca^{2+} transients was measured. Rod bipolar cells that were chemically depolarized had an AUC and maximal amplitude that was significantly higher than experiments where cells were pretreated with antagonist and the vehicle treated control. Cell pretreated with xestospongin D also had significantly higher AUC and maximal amplitudes than that of the dantrolene pretreated cells and vehicle treated control.


200
100
0
Arbitrary units
***
*
50mM KCl Depolarization
50mM KCl/ Xestospongin D
50mM KCl/Dantrolene
Vehicle control

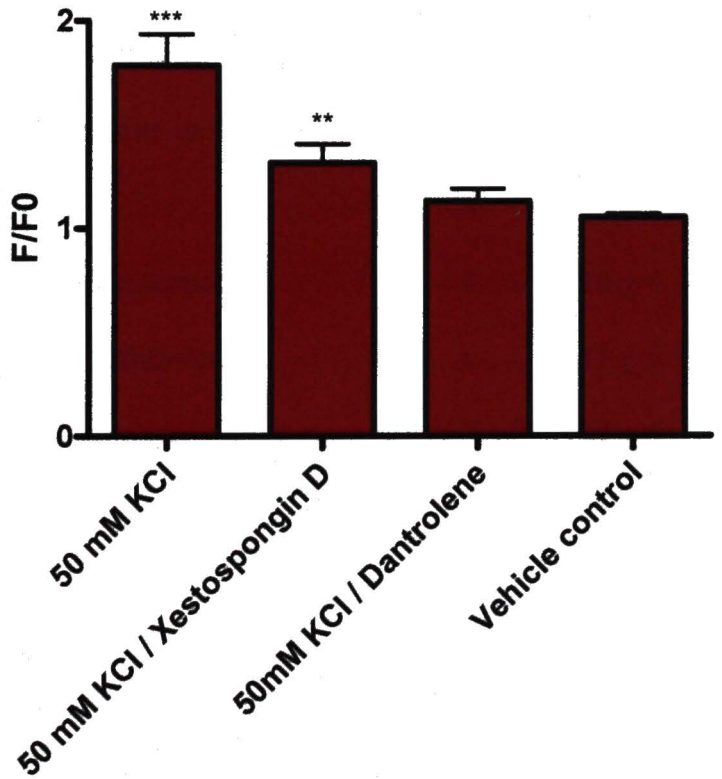

2
1
0
F/F0
***
**
50 mM KCl
50 mM KCl / Xestospongin D
50mM KCl / Dantrolene
Vehicle control

Table 1. Summary of the Pharmacologic Modulation of RyR Responses in Rod Bipolar Cells

Rod bipolar cell axon terminal F/F0 Max	**n**	**F/F0**	**SL**
RyR stimulation with caffeine in Ca^{2+} media	8	1.84 ± 0.16	***
RyR stimulation with caffeine in Ca^{2+} free media	9	1.29 ± 0.09	**
50 mM KCl stimulation	8	1.79 ± 0.15	***
50 mM KCl stimulation/Xestospongin	6	1.30 ± 0.08	**
50 mM KCl stimulation/Dantrolene	6	1.10 ± 0.03	ns
Vehicle control	7	1.05 ± 0.02	ns

Rod bipolar cell axon terminal AUC	**n**	**AUC**	**SL**
RyR stimulation with caffeine in Ca^{2+} media	8	120 ± 29	**
RyR stimulation with caffeine in Ca^{2+} free media	9	56 ± 10	ns
50 mM KCl stimulation	8	154 ± 12	***
50 mM KCl stimulation/Xestospongin	6	76 ± 4	*
50 mM KCl stimulation/Dantrolene	6	51 ± 5	ns
Vehicle control	7	48 ± 5	ns

Listed are the results from different experimental conditions using RyR agonist, caffeine in medium with and without Ca^{2+} and chemical depolarization in the presences of antagonist for the store operated receptor channels. For agonist treatment, experiments were carried out in the presence and absence of Ca^{2+} and compared against a vehicle treated control. Experiment performed in medium with Ca^{2+} had significantly higher amplitudes and AUC when stimulated with caffeine. For chemical depolarization, cells had been preincubated with either the IP_3R antagonist Xestospongin D or the RyR antagonist dantrolene, or given no treatment. When cells were preincubated with antagonist, there was a significant decrease in maximal amplitude and AUC with dantrolene pretreated cells demonstrating the greatest decrease in these parameters.

CHAPTER V

DISCUSSION AND FUTURE DIRECTIONS

The goal of this dissertation was to determine the role of metabotropic glutamate receptors (mGluRs), inositol-1,4,5,-trisphosphate receptors (IP_3Rs) and ryanodine receptors (RyRs) in increasing intracellular Ca^{2+} levels in retinal neurons as related to signaling and neurotransmitter release. In particular, we focus on the influence of these receptors and their upstream effectors and the manner that they regulated cytosolic Ca^{2+} concentrations. Our data demonstrated that activation of mGluR8 in photoreceptor cells decreased cytosolic Ca^{2+} concentrations, and that the decrease in cytosolic Ca^{2+} occurred via the $\beta\gamma$ subunit of the G-protein, inhibiting voltage gated calcium channels. We also showed that activation of the Group I mGluRs (mGluR1/5) increased cytosolic Ca^{2+} concentrations in rod bipolar cells via activating IP_3Rs. This also enabled us to determine the contribution of IP_3Rs when differentially expressed to the function of rod bipolar cells in the retina. We also studied the influence of RyRs in rod bipolar cells, its differential expression and its contribution to intracellular Ca^{2+} concentration.

Together these studies will help us understand the nature of intracellular Ca^{2+} signaling in retinal neurons, giving us insight into neurotransmitter release, and the visual cascade, as well as providing us with possible mechanism by which dysfunctional Ca^{2+} homeostasis may be implicated in neurodegenerative diseases such as retinitis pigmentosa, age-related macular degeneration, and glaucoma, as well as other neurological diseases where Ca^{2+} dyshomeostasis have been shown to be involved.

The versatility of Ca^{2+} as an intracellular signaling molecule has been demonstrated by its ability to regulate a plethora of cellular functions (Berridge et al., 2000). This diversity of regulatory pathways has lead research to focus on understanding how Ca^{2+} is able to regulate such a multitude of physiological processes within cells. This versatility of Ca^{2+} signals is thought to arise from cells have different mechanisms in place to decode the Ca^{2+} input in terms of spatiotemperal interpretation, the speed of the signal and the amplitude (Berridge et al., 2003).

As previously stated, Ca^{2+} derived from external sources and intracellular stores such as the ER play a very important role in increasing intracellular Ca^{2+} levels which can function as a Ca^{2+} signaling mechanism. Ca^{2+} release from intracellular stores such as the ER occurs through two predominant protein channels, IP_3Rs and RyRs. These channels are similar in that they share analogous molecular and physiological properties, and pharmacological characteristics (Furuichi et al., 1989; Mignery et al., 1989). These channels are regulated by similar factors such as ATP, phosphorylatory proteins, pharmacological agents, associated proteins, and Ca^{2+} itself (Takasago et al., 1991; Bezprozvanny and Ehrlich, 1993; Coronado et al., 1994; Meissner, 1994; Cardy et al., 1997; MacKrill, 1999; Koulen and Thrower, 2001; Thrower et al., 2001). By determining the specific influence of the Ca^{2+} release associated with the specific channels, we could further understand the role of these channels in neurotransmitter release and vision research.

Rehashing on the role of ON-bipolar cells and their role in the visual cascade, it is important to revisit the relationship between the Group I mGluRs and mGluR6.

Photoreceptors release the neurotransmitter glutamate at a high rate in dark conditions that hyperpolarizes ON-bipolar cells when mGluR6 is activated. Activation of mGluR6 activates a G-protein and phosphodiesterase (PDE) leading to the hydrolysis of cGMP ultimately resulting in the closing of cGMP-gated ion channels that reside on the plasma membrane (Nakanishi et al., 1994). This results in a reduction of cGMP-activated conductance that causes the hyperpolarized state in the dark. But as noted before, (Shiells and Falk, 1999; Nawy, 2000; Nawy, 2004) cytosolic Ca^{2+} concentration is vital for regulation of these ion channels, thus they may play an essential role in the mGluR6-mediated synchronization of the ON-pathway's release of visual neurotransmitter in response to changes in lighting conditions.

As hyperpolarization is taking place within these cells, ON-bipolar cell also express the Group I mGluRs (Koulen et al., 2005) which may potentially be involved in a further hyperpolarization of these neurons. mGluR1/5 initiates Ca^{2+} signaling through G-protein-dependent intracellular Ca^{2+} release via phospholipase C (PLC) and inositol-1,4,5-trisphosphate (IP_3) activation (Pin and Duvoisin, 1995; Hermans and Challiss, 2001) which increases intracellular Ca^{2+} concentrations. mGluR1/5, being glutamate receptors, are simultaneously being activated in the dark when glutamate levels are increased, causing the release of Ca^{2+} from store operated channels on the ER via IP_3Rs.

Apart from the Group I mGluR/IP_3R pathway, the Group I mGluR indirectly initiates hyperpolarization via activation of Ca^{2+}-activated K^+ channels that allows K^+ to leave the cytoplasm. The Ca^{2+} influx that activates the Ca^{2+}-activated K^+ channels stem from the mGluR1/5 initiated IP_3 release (Fiorillo and Williams, 1998; Gebremedhin et

al., 2003). This synchronized regulation of ions, may potentially act as another method of fine tuning the Ca^{2+} signaling. It should therefore be distinguished that the behavior of the group I mGluRs is dependent on the system and physiological environment since they are also capable of depolarizing neuronal cells (Pin and Acher, 2002).

Within the central nervous system (CNS) of which the retina belongs, neurons are capable of being active without any direct synaptic input, thus demonstrating spontaneous activity. This spontaneous activity can be visualized during calcium imaging experiments where even before stimulation there is a constant influx and efflux of Ca^{2+} ions into the cytosol of rod bipolar cells. The fluctuation seen is not very robust thus when cells are actually stimulated, we are capable of distinguishing a true agonist response versus spontaneous Ca^{2+} fluctuation. To account for this activity during experimental conditions, control experiments were performed where the treatment-vehicle solutions were used to activate cells. This treatment did not generate any significant changes in Ca^{2+} activity with rod bipolar cells. It should also be noted that this spontaneous activity is very important for neuronal function within neurons (Llinas, 1988).

Studies on isolated goldfish Mb1 type bipolar cells have shown them capable of generating Ca^{2+}-dependent spontaneous regenerative membrane oscillations through Ca^{2+} influx via L-type voltage gated Ca^{2+} channel activity (Burrone & Lagnado, 1997; Zenisek & Matthews, 1998), where these changes in potential have been shown to be involved in bipolar cell interpretation of light stimulation (Protti et al., 2000). L-type voltage gated Ca^{2+} channel have also been shown to be expressed in mammalian bipolar cells, and in bipolar cells of lower vertebrates, along with the T-type voltage gated Ca^{2+} channel

(Lasater, 1988; Kaneko et al., 1989; Maguire et al., 1989; Pan & Lipton, 1995; de la Villa et al., 1998; Hartveit, 1999; Pan, 2000).

The release of glutamate from photoreceptor in dark condition causes hyperpolarization via activation of mGluRs on ON-bipolar cells dendrites and depolarization in light condition due to reduced glutamate release from photoreceptor cells. In the dark, when photoreceptor cells are depolarized, voltage-gated Ca^{2+} channels are activated in their synaptic terminals, allowing for the influx of Ca^{2+}, triggering glutamate release from the axon terminals.

Here we showed that voltage-gated Ca^{2+} channels on rod bipolar cells initiates the influx of Ca^{2+} from extracellular sources, which then activated RyR on intracellular stores, leading to a potentiated Ca^{2+} response. This data corroborates the fact that bipolar cells undergo graded potentials rather than full action potentials that are necessary for retinal neurons that undergo graded changes in response to light that are dependent on subtle changes in Ca^{2+} concentration.

Future directions

The mGluR8 has been shown to be an autoreceptor localized in the presynaptic area of mouse photoreceptor cells. It has been shown to decrease the activity of the L-type Ca^{2+} channels thus causing a reduction in intracellular Ca^{2+} levels of photoreceptors and Ca^{2+} is known to play a vitally important role in neurotransmitter release.

Future directions should be to establish the role of mGluR8 in neurotransmitter release and its mechanism of regulation. To determine the role of mGluR8 in

neurotransmitter release, it is important to know the subcellular localization of these receptors within the mouse photoreceptor cells. Proximity to features such as vesicle ribbons and the L-type Ca^{2+} channels in the presynaptic region is a strong indicator of the mGluR8 involvement in neurotransmitter release. To determine the sub cellular localization and interaction with synaptic structures, we can employ co-immunoprecipitation, and immunoreactive studies.

It has been also been shown that mGluR8 affects calcium levels via inactivation of L-type voltage gated Ca^{2+} channels. Literature has established that the mGluR8 is negatively coupled to adenylyl cyclase via G protein coupling. The activation of mGluR8 and its interaction with the L-type calcium channels in photoreceptors occur via the $\beta\gamma$ subunit of a specific unknown G protein. The characterization of this unknown G protein can unlock pathways that this protein maybe involved in within the retina. Characterization can be done using immunocytochemistry, immunohistochemistry, gel electrophoresis and western blot, and cross-linking experiments. Functional assays such as patch clamping and/or Ca^{2+} imaging experiments can also be used to establish the relationship between mGluR8, L-type Ca^{2+} channels, and G_t.

Since it has been established that Ca^{2+} is essential for the release of neurotransmitter from vesicles within a synapse, and that the mGluR8 is known to reduce intracellular Ca^{2+} levels in rod photoreceptor cells, we can hypothesize that the activation of mGluR8 reduces the levels of glutamate being released from mouse photoreceptor cells. These receptors may therefore provide a mechanism of regulating glutamate release, by modulating calcium levels at the synaptic region based on the receptor

activity. Using optical imaging, activation and inactivation of the mGluR8 and the L-type Ca^{2+} channels using agonist and antagonist of these proteins, and using fluorometric probes to measure glutamate levels during different conditions, we can determine the specific relationship between mGluR8 and neurotransmitter release.

Conclusion

In conclusion, we have demonstrated that retinal neurons have multiple mechanisms in place for regulating intracellular Ca^{2+} concentrations implying that there are multiple ways by which these neurons modulate intracellular Ca^{2+} signaling and neurotransmitter release. We first described that activation of mGluR8 in photoreceptor cells decreased cytosolic Ca^{2+} concentrations via inhibition of voltage gated Ca^{2+} channels (Fig. 1). This is very important with regards to neurotransmitter release from photoreceptor cells since mGluR8 is an autoreceptor residing on the presynaptic terminal.

We also showed that activation of the Group I mGluRs (mGluR1/5) increased cytosolic Ca^{2+} concentrations in rod bipolar cells via activating IP_3Rs (Fig. 2), highlighting the relationship between glutamate release from photoreceptor cells and rod bipolar cell $[Ca^{2+}]_c$. This enabled us to determine the contribution of IP_3Rs when differentially expressed to the function of rod bipolar cells in the retina.

We also studied the influence of RyRs in rod bipolar cells, its differential expression and its contribution to $[Ca^{2+}]_c$. This study provided insight into the Ca^{2+} releasing pathway in rod bipolar cells where we were able to show the importance of intracellular Ca^{2+} stores during depolarization of these cells. Ultimately, these studies will help in the understanding of the visual pathway and fine tuning of Ca^{2+} signaling with

respect to neurotransmitter release. They also provide potential targets for therapeutic treatments in diseases of the retina involving Ca^{2+} dysfunction.

CHAPTER VI

APPENDIX

Figure 1. mGluR8 regulates intracellular Ca^{2+} level by inactivating voltage gated Ca^{2+} channels on the plasma membrane of photoreceptor cells

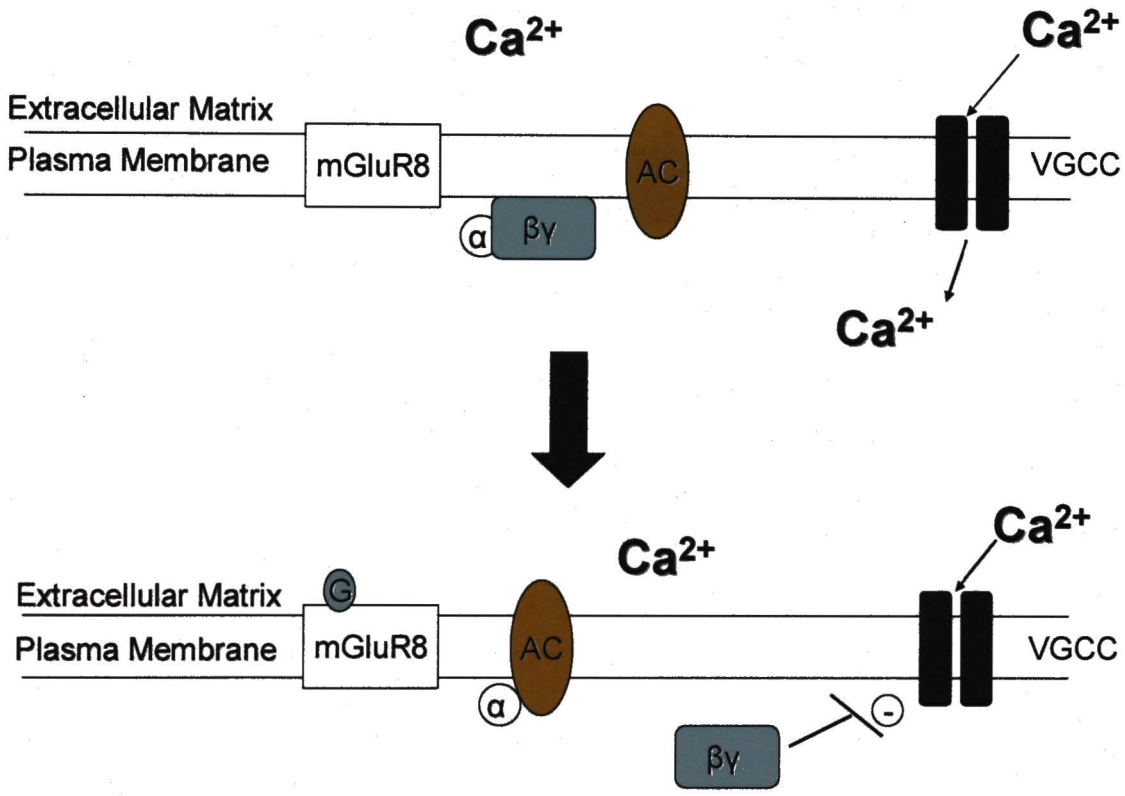
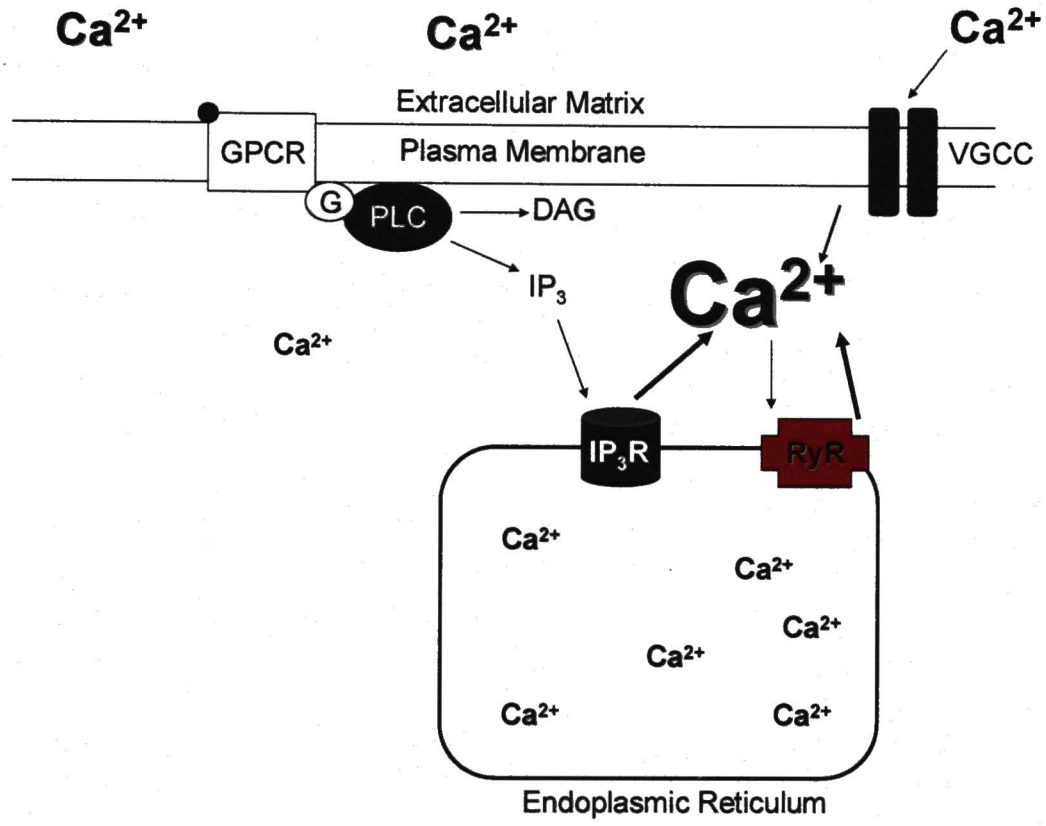


Figure 2. IP_3Rs and RyRs contributes to the Ca^{2+} signaling in rod bipolar cells



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