Progesterone Potentiates IP₃-Mediated Calcium Signaling Through Akt/PKB

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Key Words

Gonadal steroid hormone • Central nervous system • Neuron • Retina • Electrophysiology • Kinase

the gain of the Ca²⁺ second messenger system as a signaling gatekeeper for neuronal activity the present study identifies a novel pathway for interaction of the endocrine and central nervous system.

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Abstract

The activity of cells critically depends on the control of their cytosolic free calcium ion (Ca²⁺) concentration. The objective of the present study was to identify mechanisms of action underlying the control of the gain of intracellular Ca²⁺ release by circulating gonadal steroid hormones. Acute stimulation of isolated neurons with progesterone led to IP₃R-mediated Ca²⁺ transients that depend on the activation of the PI3 kinase/Akt/PKB signaling pathway. These results were confirmed at the molecular level and phosphorylation of IP₃R type 1 by Akt/PKB was identified as the mechanism of action. Hence, it is likely that circulating gonadal steroid hormones control neuronal activity including phosphorylation status through receptor- and kinase-mediated signaling. With a direct control of

Introduction

Transient global or local changes in the intracellular Ca²⁺ concentration mediate critical signaling processes in neurons [1-3]. Inositol 1, 4, 5-trisphosphate (IP₃)-gated Ca²⁺ channels / IP₃ receptors (IP₃R) control the release of Ca²⁺ from intracellular stores and thus, contribute to these transients. The G protein-mediated generation of IP₃ via phospholipase C (PLC) interfaces the stimulation of receptors on the plasma membrane with intracellular Ca²⁺ signaling (reviewed in [4]), ultimately controlling a plethora of cellular processes [3, 5-7].

IP₃Rs are localized in neurons of the mammalian retina including bipolar cells [8-13] identifying potential

roles for these receptors in the activity of these retinal neurons [13]. Such potential functions had been confirmed through indirect physiological evidence that indicates a central role for IP₃Rs in intracellular Ca²⁺ signaling of retinal neurons [14-22]. Mammalian rod bipolar cells were chosen for their well characterized expression [8-13, 23, 24] and functional utilization of IP₃-mediated intracellular Ca²⁺ signaling [13, 25-32]. The functional relevance of IP₃R controlled Ca²⁺ stores [8, 10-13] in rod bipolar cells is complemented by the expression of upstream signaling elements, namely group I metabotropic glutamate receptors (mGluRs) [1, 33], and the presence of PLC activity [34] in these cells. Ca²⁺ signaling via group I mGluRs in rod bipolar cells [1, 33], has been shown to be differentially controlled by the activity of distinct types of IP₃R [13]. Based on their characteristic morphology and physiology, rod bipolar cells, as the first interneurons of the retina that process neuronal information in both synaptic layers of the neural retina [23, 24], serve also as a model for other CNS interneurons [25-32].

Progesterone receptors have also been identified in the mammalian retina [35]. Similarly, downstream signaling proteins potentially involved in mediating progesterone activity were detected in the retina: ERK/ MAP Kinase [36-44], Raf [41, 45, 46], MEK [40-45], and Akt [47-53].

The present study addresses potential crosstalk between both signaling pathways, IP₃R mediated Ca²⁺ signaling and progesterone-mediated activation of intracellular signaling pathways. In particular, mechanistic aspects of how circulating gonadal steroid hormones affect biophysical properties of IP₃Rs at the molecular and cellular level through kinase signaling cascades were investigated.

Materials and Methods

Experimental procedures described in the present study were performed in accordance with guidelines for the welfare, care and use of experimental animals issued by the National Institutes of Health, and UNTHSC and are in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Cell isolation, immunochemistry and phosphorylation experiments

Isolation of rod bipolar cells from outbred Swiss Webster mouse (Harlan, Indianapolis, Indiana) retina using a combination of enzymatic tissue digestion and mechanical trituration was performed as described previously [3, 54, 55]. Morphology and immunoreactivity of the dissociated cells were used for identification [13, 54, 55]. Previous reports had shown that both

subcellular morphology and functions are maintained in these acutely, dissociated isolated rod bipolar cells [13, 56-61]. We had shown previously for this cellular preparation that sub-structural distribution of key signaling proteins is also preserved after mechanical and enzymatic dissociation of rod bipolar cells and that redistribution of proteins does not occur during or after dissociation [13]. Cells were seeded onto poly-D-lysine pre-coated coverslips and processed for immunocytochemistry or imaging of intracellular Ca²⁺ concentrations using fluorescence microscopy. For immunochemistry aldehyde fixed cells were incubated for two hours each in blocking solution, primary and subsequently secondary antibody solutions as detailed previously [3, 54, 55]. Immunoreactivity and primary antibodies (see details below) were detected with goat anti-mouse or anti-rabbit IgG secondary antibodies coupled to the fluorescent dyes Alexa Fluor® 488 and Alexa Fluor® 594 (Molecular Probes, Inc., Eugene, OR, diluted 1:500) and standard immunofluorescence microscopy [13, 54, 55].

A previously described immunopanning technique [13] for the bulk purification of ON-bipolar cells of which rod bipolar cells make up approximately 64% (remainder ON-cone bipolar cells) [62, 63] was used to obtain cells for immunoblot analyses and for isolating endoplasmic reticulum (ER). Briefly, dissociated retina cells were incubated in vials (Corning, Acton, MA) that had been coated with a specific antibody directed against metabotropic glutamate receptor 6 (mGluR6) (rabbit polyclonal antibody directed against the N-terminal extracellular region of mGluR6; Novus Biologicals, Littleton, CO; 1:500). Isolated adherent ON bipolar cells were separated by centrifugation and processed for either immunochemistry blot analyses or isolation of the ER. Standard immunoblotting methods described previously [13] were used to identify and/or semiquantitatively compare immunoreactivities for signaling proteins (antibodies, see details below). Proteins from isolated ON bipolar cells were immunoprecipitated by incubation with specific antibodies (see details below) for 16 h and were isolated using protein A-agarose (Sigma-Aldrich, St. Louis, MO), washed and processed with SDS-PAGE for further immunoblotting analyses. For *in vitro* analyses of Akt phosphorylation, immunoprecipitated proteins bound to protein A-agarose were incubated in 0.05 M Tris / HCl buffer, pH 7.4 containing recombinant, constitutively active Akt (0.625 µg/mg lysate protein; catalog #SE248, BIOMOL International L.P., Plymouth Meeting, PA), 100 µM ATP and 5 mM MgCl₂ for 3 h at 37°C., washed and processed with SDS-PAGE for further immunoblotting analyses. For some experiments, immunoprecipitated proteins were co-incubated with a peptide mimicking the Akt phosphorylation site and representing a substrate for Akt competing with cellular substrates (ARKRERTYSFGHHA, AKTide-2T, EMD Biosciences). A peptide lacking the serine phosphorylation site (ARKRERAYAFGHHA, AKTide-SA, EMD Biosciences) was used as control for these experiments.

Antibodies

Immunoreactivities for IP₃R types, progesterone receptors and intracellular signaling proteins and their targets were identified with specific antibodies (IP₃R type 1: EMD

Biosciences, La Jolla, CA, rabbit polyclonal, for Western blotting (WB), 1:1000, for immunocytochemistry (ICC), 1:100 [13, 64, 65]; Chemicon, Temecula, CA, rabbit polyclonal, AB9072, for WB and ICC, 1:1000; IP₃R type 2: EMD Biosciences, rabbit polyclonal, for WB, 1:1000 for ICC, 1:100 [13, 66]; Chemicon, rabbit polyclonal, AB9074, for WB and ICC, 1:1000; IP₃R type 3: BD Biosciences Pharmingen, San Diego, CA, mouse monoclonal, for WB, 1:1000, for ICC, 1:100 [13, 66]; Chemicon, rabbit polyclonal, AB9076, for WB and ICC, 1:1000; phosphoserine: Biomed, Foster City, CA, mouse monoclonal, V3156, for WB, 13 µg/ml; phospho-threonine: Chemicon, rabbit polyclonal, AB1607, for WB, 2 µg/ml; phospho-serine/threonine Akt substrate, i.e. peptides and proteins with a phospho-Ser/Thr residue and a preceding Lys/Arg at positions -5 and -3, independent of other neighboring amino acid residues: Cell Signaling Technology, Inc., Danvers, MA, rabbit polyclonal, catalog #9611, for WB, 1:1000; pan-actin: Biomed, rabbit polyclonal, V10275, for WB, 0.5 µg/ml; progesterone receptor: Santa Cruz Biotechnology, Inc., Santa Cruz, CA, rabbit polyclonal, PR (C-19): sc-538, for ICC, 1: 500; phospho-p44/42 MAP Kinase (Thr202/Tyr204): Cell Signaling Technology, Danvers, MA, rabbit polyclonal, #9101, for ICC, 1:300, for WB, 1:1000; p44/42 MAP Kinase: Cell Signaling Technology, rabbit polyclonal, #9102, for ICC, 1:100, for WB, 1:1000; phospho-Akt (Ser473): Cell Signaling Technology, rabbit polyclonal, #4058, for ICC, 1:50, for WB, 1:1000.

In addition, antibodies specific for the characterization and identification of intact ON and/or rod bipolar cells were used (mGluR1: Chemicon, rabbit polyclonal, for ICC, 0.2 µg/ml [1, 13]; mGluR5: Chemicon, rabbit polyclonal, for ICC, 1 µg/ml [1, 13]; mGluR6: Novus Biologicals, rabbit polyclonal, for immunopanning, 1:500; protein kinase C α : Seikagaku, Tokyo, Japan, mouse monoclonal, for ICC, 1:100 [13, 55].

Imaging of intracellular Ca²⁺ concentrations using fluorescence microscopy

Previously described experimental protocols [13, 54, 67, 68] were used for calcium imaging: Isolated retinal neurons including rod bipolar cells were plated as described above and maintained in L-15 medium (Leibovitz medium, Sigma-Aldrich) for several hours after dissociation.

After incubation in cell-permeant fluo-3 (4 µM at 37°C for 30 min; fluo-3-acetoxymethylester; Molecular Probes, Eugene, OR), cells were washed and used for recording. Using 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) changes in fluo-3 emission fluorescence intensity of individual loaded cells over time was determined. A gravity-fed perfusion system (flow rate, 1 ml / minute) was used to perfuse cells with fresh L-15 medium and compounds dissolved in L-15 medium as needed for the respective experimental strategies. Changes in intracellular Ca²⁺ concentration transients of rod bipolar cells were plotted as the ratio of fluo-3 emission fluorescence intensity (F) over the 10s average of the baseline fluorescence intensity

before drug application (F₀) after image acquisition at a rate of 2-10 Hz.

Progesterone (10-100 nM; Sigma-Aldrich, St. Louis, MO), the ryanodine receptor blocker dantrolene (100 µM; Sigma-Aldrich), the IP₃R inhibitor xestospongin C (1 µM; Sigma-Aldrich), the MAPK inhibitor U0126 (10 µM), and/or the PI-3 kinase inhibitor LY294002 (15 µM) were applied either acutely or administered as a pre-incubation (in L-15 medium at 37°C) of cells to inhibit their respective targets. These concentrations were chosen based on previously published studies that had investigated the respective concentration response relationships and that had identified the chosen concentrations as optimal [69-71]. With respect to the concentrations of inhibitors, concentrations were chosen that were approximately 10-fold the concentration of the reported EC₅₀ or K_i values respectively [72]. Similar to the incubation with agonists or inhibitors, for some experiments cells were pre-incubated for 1 hr with a peptide mimicking the Akt phosphorylation site and representing a substrate for Akt competing with cellular substrates (ARKRERTYSFGHHA, AKTide-2T, EMD Biosciences) in the presence of Chariot™ protein delivery reagent (Active Motif, Carlsbad, CA) mediating translocation of the peptide to the cytosol. A peptide lacking the serine phosphorylation site (ARKRERAYAFGHHA, AKTide-SA, EMD Biosciences) was used as control for these experiments. (S)-3,5-Dihydroxyphenylglycine (S-DHPG, Sigma-Aldrich; 0.1 - 250 µM), an agonist for group I mGluRs, receptors that are coupled to IP₃ signaling [73, 74], was used in bath-applied bolus stimulation to generate IP₃-mediated intracellular Ca²⁺ concentration transients. At the end of experiments, intactness of intracellular Ca²⁺ stores was tested by bath-application of 10 µM thapsigargin (EMD Biosciences), which inhibits sarcoplasmic and endoplasmic reticulum Ca²⁺ ATPases [75] and thereby is useful to assess overall filling of intracellular Ca²⁺ stores. To exclude and evaluate the contribution of extracellular Ca²⁺ to the measured intracellular Ca²⁺ concentration transients, L-15 medium lacking Ca²⁺ and containing 5 mM EGTA to buffer potentially present Ca²⁺ impurities was used alternatively to L-15 medium (contains 1.25 mM Ca²⁺). Experiments performed under extracellular Ca²⁺-free conditions involved a 2 min pre-equilibration with the Ca²⁺-free L-15 medium before application of pharmacological agents. This time frame was chosen to eliminate residual Ca²⁺ in the extracellular space without significantly depleting Ca²⁺ stores.

For each experimental condition at least 5 cells were recorded and analyzed in triplicate. Statistical analyses comprised standard one way or multiple ANOVA when comparing parametric populations.

Single channel electrophysiology using the bilayer lipid membrane system

mGluR6 immunopanning (see above) was used to isolate ON-bipolar cells that had been exposed to vehicle control substances or pretreated with progesterone and/or pharmacological modulators. ER vesicles were prepared from these isolated cells and incorporated into bilayer lipid membranes as described previously [13, 67, 68]. Activity of single IP₃Rs contained in these ER vesicles was measured with

250 mM HEPES-Tris solution, pH 7.35 on the cytosolic side and 250 mM HEPES, 55 mM Ba(OH)₂ solution, pH 7.35 on the luminal side of the channel. To identify IP₃R activation by IP₃ and inactivation by heparin (50 mg/l; Sigma-Aldrich) or xestospongin C (1 μM) was pharmacologically induced. The dependence of IP₃R activity on varying concentrations of both cytosolic free Ca²⁺ and IP₃ was determined. RyRs were not detected in ER preparations of ON-bipolar cells as reported previously [13]. Single channel activity was acquired using voltage-clamp conditions, filtering at 3 kHz and digitization with a workstation for planar lipid bilayer (Warner Instruments, Inc., Hamden, CT). Biophysical parameters such as mean dwell time, current amplitude and open probability were acquired and analysed with pClamp version 8.1 software (Axon Instruments, Union City, CA). These biophysical parameters and the activity dependence on cytosolic free Ca²⁺ and IP₃ were compared to published reference data [13, 76, 77], reviewed in [2, 78, 79] to identify IP₃R subtypes biophysically and pharmacologically.

Three or more independent data sets were obtained for each experimental condition. Statistical analyses were performed with standard one way or multiple ANOVA when comparing parametric populations.

Results

In the present study, the distribution of proteins potentially related to progesterone signaling in rod bipolar cells was assessed and functional consequences of progesterone signaling on the activity of IP₃R and IP₃R-mediated neuronal Ca²⁺ signaling were determined. This was achieved using a multidisciplinary approach comprising immunochemical methods, Ca²⁺ imaging and single channel electrophysiology.

The co-distribution of immunoreactivity for the progesterone receptor and for IP₃R type 1 was determined using specific antibodies and immunocytochemical fluorescent stainings of acutely isolated mouse rod bipolar cells (figure 1). Immunoreactivity for type 1 IP₃R was found throughout the cell and in high density in the soma and axon terminal (figure 1a). Similarly, immunoreactivity for the progesterone receptor was found throughout the neuron with higher labeling intensity in the soma and axon terminal system (figure 1b) compared to a control with secondary antibody alone that showed no obvious signal (figure 1c). This apparent co-distribution of the IP₃R and the progesterone receptor in the same subcellular regions provides the potential molecular substrate for signaling cascades originating from steroid hormone signaling and an interface between hormone signaling and IP₃-mediated

Fig. 1. Immunoreactivity for IP₃R and PR in acutely isolated mouse rod bipolar cells. After fixation, freshly dissociated rod bipolar cells were stained for IP₃R type 1 (a), PR (b) and with secondary antibody (goat anti rabbit IgG) alone as a control (c). Immunoreactivity for both receptors was found throughout the neuron with strong labeling of the soma and axon terminal system. Differential interference contrast images to the right of each fluorescence photomicrograph identify major structures of the neuronal morphology, dendrites, soma, axon, and axon terminal system.

Ca²⁺ signaling. Therefore, we subsequently investigated effects of progesterone on general and IP₃-mediated Ca²⁺ signaling (figure 2) using acutely isolated rod bipolar cells ester-loaded with the Ca²⁺ indicator dye fluo-3 and monitoring of emission fluorescence as a measure for intracellular Ca²⁺ concentrations. When isolated rod bipolar cells were stimulated with progesterone a transient rise in cytosolic Ca²⁺ was observed after a delay of 4-5 minutes (figure 2a, b). Using well-documented control assays for optical imaging of intracellular Ca²⁺ concentrations and pharmacological tools, the contribution of extracellular versus intracellular sources of Ca²⁺ (figure 2c, d), of different types of intracellular Ca²⁺ channels (figure 2d, e) and of kinase signaling pathways (figure 2f), to the Ca²⁺ transient was determined. The progesterone-induced Ca²⁺ transients were detected predominantly in the portion of the cell located in the outer retina, namely the soma, and persisted for several minutes (figure 2a, b). When extracellular Ca²⁺ was removed from the perfusion medium or when plasma membrane Ca²⁺ channels were blocked with non-specific (Co²⁺) or L-type Ca²⁺ channel specific blockers (nifedipine), the progesterone-induced Ca²⁺ transient remained unaltered indicating a mechanism of action mediated by release of Ca²⁺ from intracellular stores (figure 2c). This was further

Fig. 2. Stimulation of mouse rod bipolar cells with progesterone induces a delayed-onset, long-lasting Ca^{2+} transient in the soma. Mouse rod bipolar cells were ester-loaded with fluo-3 and changes in emission fluorescence were monitored. In panel (a) images taken at 150 s time intervals after addition of 100nM progesterone (time point: 0 s) of a representative experiment are shown. A progesterone-induced Ca^{2+} transient appeared predominantly in the soma and primary dendrites and lasted several minutes. In panel (b), changes in fluo-3 emission fluorescence of the soma region of the mouse rod bipolar cell shown in (a) are plotted over time. The progesterone-induced signal was not affected by the removal of extracellular Ca^{2+} or block of calcium channels on the plasma membrane (c). However, pre-stimulation with an IP_3 -generating metabotropic glutamate receptor type 1 ligand (S-DHPG) leading to a depletion of intracellular Ca^{2+} stores prevented the formation of the progesterone-induced Ca^{2+} transient in the presence and absence of extracellular Ca^{2+} (d). Blockade of intracellular Ca^{2+} channels has differential effects on the progesterone-induced Ca^{2+} transients in mouse rod bipolar cells (e). Whereas pre-incubation of cells with the RyR inhibitor, dantrolene, did not affect progesterone's action, pre-incubation with the IP_3R inhibitor xestospongine abolished progesterone-mediated Ca^{2+} transients (e). Preincubation of mouse rod bipolar cells with compounds affecting intracellular signaling pathways indicate a linkage of progesterone-induced Ca^{2+} signaling to the PI-3 kinase / Akt kinase-signaling cascade (f). Whereas pre-incubation with the MAPK inhibitor U0126 (10 μM) did not affect progesterone-induced intracellular Ca^{2+} transients, the PI-3 kinase inhibitor LY294002 (15 μM) reduced the Ca^{2+} signal to background levels. Similarly, pre-incubation of the cells with a peptide mimicking the Akt phosphorylation site and representing a substrate for Akt competing with cellular substrates, in the presence of ChariotTM protein delivery reagent (Active Motif, Carlsbad, CA) mediating translocation of the peptide to the cytosol, led to a complete reduction of the progesterone-mediated induction of Ca^{2+} transients.

corroborated by the fact that pre-stimulation of the IP_3R -controlled Ca^{2+} signaling pathway through activation of the IP_3 -generating metabotropic glutamate receptor type

1 (mGluR1) rendered the cells unresponsive to an immediately subsequent progesterone stimulus (figure 2d). Alternatively, pre-stimulation with progesterone

Fig. 3. Phosphorylation of IP₃R by Akt after progesterone-mediated Akt activation. Western blot showing the expression of the activated form of Akt/PKB, Akt phosphorylated at amino acid S473 (a). Enzymatically isolated mouse rod bipolar cells were homogenized after 30 min treatment with vehicle control, 100 nM progesterone or 100 nM progesterone in the presence of the PI-3 kinase inhibitor LY294002 (15 μ M). An increase in the activated phosphorylated form of Akt/PKB was observed after treatment with progesterone and was LY-294002-sensitive (a). Detection of immunoprecipitated IP₃R type 1 from mouse rod bipolar cells that had been pre-incubated with the same compounds as in panel (a) showed an increase in phosphorylated serine, but no significant changes in phosphorylated threonine levels in bands with the approximate molecular weight of IP₃R type 1 (250 kDa bands) (b). (c) Immunoprecipitated IP₃R type 1 from 400 μ g of untreated mouse rod bipolar cell lysate was incubated for 3 h with 250 ng of Akt subjected to PAGE and immunoblotted with the anti-phosphorylated serine/threonine Akt substrate antibody. Immunoreactivity was significantly reduced when samples were co-incubated with Akt and a peptide mimicking the Akt phosphorylation site, representing a substrate for Akt competing with cellular substrates or when Akt was absent (c). In a schematic diagram of the IP₃R type 1, both MAPK (■) and Akt (★) phosphorylation sites located on cytoplasmic domains of the receptor can be found (d).

abolished the Ca²⁺ transient of an immediately following stimulation of mGluR1 via its specific agonist S-DHPG indicating that both signals derived their Ca²⁺ from the same intracellular stores and that depletion of stores by one stimulus affects the efficacy of a subsequently administered, separate and different stimulus (figure 2c). These mechanisms were again independent of the presence of extracellular Ca²⁺ (figure 2c). Whereas blocking of Ca²⁺ channels located on the plasma membrane did not affect the progesterone-induced Ca²⁺ transients in mouse rod bipolar cells (figure 2c, d), pharmacological inhibition of intracellular Ca²⁺ channels revealed a distinct signaling profile (figure 2e). Pre-incubation of cells with dantrolene, a RyR inhibitor, had no effect on the progesterone-mediated Ca²⁺ transient. Pre-incubation with the IP₃R inhibitor xestospongin, however, abolished the signal (figure 2e). To further

analyze the potential mechanisms of action involved in this progesterone-mediated modulation of IP₃R signaling, the involvement of two major kinase signaling pathways typically associated with progesterone signaling was determined (figure 2f). When mouse rod bipolar cells were pre-incubated with the MAPK inhibitor, U0126, amplitude and kinetics of the progesterone-induced intracellular Ca²⁺ transients were unaffected (figure 2f). However, the PI-3 kinase inhibitor LY294002 acting upstream of Akt/PKB signaling abolished the progesterone-induced Ca²⁺ signal (figure 2f). With a different approach, similar results, i.e. dependence of the progesterone-induced intracellular Ca²⁺ transient on activity of the Akt/PKB signaling pathway, were obtained: Introduction of a peptide mimicking the Akt phosphorylation site and representing a substrate for Akt, competing with cellular substrates by means of the ChariotTM protein delivery reagent (Active

Fig. 4. Single channel activity of IP₃R from the ER of mouse rod bipolar cells that had been pre-incubated with vehicle (a), 100nM progesterone (b), 100nM progesterone and 10μM MAPK inhibitor U0126 (c), 100nM progesterone and 15μM Akt inhibitor LY294002 (d), or a peptide mimicking the Akt phosphorylation site and representing a substrate for Akt competing with cellular substrates, in the presence of Chariot™ protein delivery reagent (Active Motif, Carlsbad, CA) mediating translocation of the peptide to the cytosol (e). Recording of single IP₃R channels was performed with 0.5μM free Ca²⁺, 500μM ATP and 10μM ruthenium red, a RyR blocker, were added to the cytoplasmic face of the channels. Recordings were performed with Ba²⁺ as the current carrier. Single channel zero current baseline levels are indicated by bars to the right of individual traces with channel openings plotted as downward deflections. Traces of representative experiments are shown in a-e. Pre-incubation with progesterone led to significantly increased channel activity (b) when compared to control conditions (a). Co-incubation of cells with progesterone and a MAPK inhibitor did not change the progesterone-mediated increase in channel activity (c); whereas co-incubation of mouse rod bipolar cells with progesterone and an Akt inhibitor (d) or with a peptide competing as an Akt substrate leading to Akt inhibition (e) produced channel activity comparable to baseline levels (a). Panel (f) summarizes changes in open probability and single channel conductance of mouse rod bipolar cell IP₃R isolated from different experimental conditions. Dependence of channel activity on cytosolic free Ca²⁺ concentrations (g) and open probability (h) after pre-incubation of mouse rod bipolar cell IP₃Rs with 100nM progesterone (△) or vehicle (▲) were determined. While progesterone pre-incubation did not influence the overall cytosolic Ca²⁺ dependence of IP₃Rs from mouse rod bipolar cell which showed typical IP₃R type 1 behavior, open probability was increased at all physiologically relevant Ca²⁺ concentrations (g). At the same time, experiment analyzing the IP₃ dependence of the receptors (h) indicate that pre-incubation of mouse rod bipolar cell IP₃Rs with 100nM progesterone reduced the EC₅₀ from 205 ± 11nM in vehicle controls (▲) to 124 ± 9nM (△).

Motif, Carlsbad, CA) that induced translocation of the peptide to the cytosol also completely abolished

progesterone-mediated Ca²⁺ transients (figure 2f). These two sets of data that were obtained by measuring the

contribution of intracellular signaling pathways, dependence of the progesterone-mediated Ca^{2+} signal on IP_3R -mediated Ca^{2+} release and control of the signal by the Akt/PKB signaling pathway, suggest a link of progesterone-induced Ca^{2+} signaling to the PI-3 kinase- / Akt kinase-signaling cascade controlling the gain of IP_3R -mediated intracellular Ca^{2+} release activity (figure 2f). This prompted us to further determine the molecular identity of this interaction between IP_3R -mediated Ca^{2+} signaling and activation of the PI3 kinase/Akt/PKB signaling pathway. Using epitope-specific immunoblotting assays, an increase in the activated form of Akt/PKB, Akt phosphorylated at amino acid S473, was detected in enzymatically isolated mouse rod bipolar cells after exposure to progesterone (figure 3a). This increase in immunoreactivity for active Akt was blocked with the PI-3 kinase inhibitor LY294002 (figure 3a). To determine if IP_3Rs were a potential target for Akt, type 1 IP_3Rs were immunoprecipitated from mouse rod bipolar cells that had been treated experimentally the same way as cells shown in figure 2a. An increase in immunoreactivity of the precipitate for phosphorylated serine, but not phosphorylated threonine was observed in bands with the approximate molecular weight of IP_3R type 1, 250 kDa, (figure 3b). This result was confirmed in vitro using immunoprecipitated IP_3Rs from untreated mouse rod bipolar cells and incubating them with recombinant constitutively active Akt. Experiments were performed in the presence or absence of a peptide mimicking the Akt phosphorylation site thus representing a substrate for Akt competing with cellular substrates such as the immunoprecipitated IP_3Rs (figure 3c). Incubation with Akt resulted in significant immunoreactivity for phosphoserine/threonine Akt substrate. The signal was reduced when immunoprecipitated IP_3Rs had been co-incubated with a peptide mimicking the Akt phosphorylation site and representing a substrate for Akt competing with cellular substrates (AKTide-2T) or when Akt was absent (figure 3c). This is in agreement with the presence of an Akt phosphorylation site at amino acid 2681 in the cytoplasmic C-terminal tail of the IP_3Rs (figure 3d). MAPK phosphorylation sites were also identified on IP_3R type 1, but they were not affected by progesterone signaling in the rod bipolar cells (data not shown) consistent with Ca^{2+} imaging data (figure 2f). These immunochemical data further suggested a direct functional interaction between the PI3 kinase/Akt/PKB signaling pathway stimulated by progesterone and IP_3R -mediated Ca^{2+} signaling, which prompted us to investigate IP_3R

activity at the single channel level. Using planar lipid bilayer electrophysiology, the activity of IP_3Rs in endoplasmic reticulum (ER) preparations from mouse rod bipolar cells was measured after pre-incubation with different pharmacological modulators (figure 4). Preincubation with vehicle (figure 4a) showed control baseline activity. When cells had been preincubated with progesterone, a significant increase in single channel open probability was observed (figure 4b, f) that could not be inhibited by co-preincubation with progesterone and the MAPK inhibitor U0126 (figure 4c, f). However, IP_3Rs isolated from cells that had been incubated with progesterone and the Akt inhibitor LY294002 (figure 4d), or into which a peptide mimicking the Akt phosphorylation site and representing a substrate for Akt had been introduced (figure 4e), showed no increased activity (figure 4d-f). Conductance changes were not observed under any of the described conditions and were typical for type 1 IP_3R . Recordings were performed in the presence of ruthenium red, a RyR blocker, on the cytoplasmic face of the channel and with Ba^{2+} as the current carrier to avoid modulation of channel activity by Ca^{2+} as the current carrier. Subsequently, effects of progesterone treatment on the two major regulatory components of IP_3R activity, regulation by cytosolic free Ca^{2+} and by the cytosolic IP_3 concentration, were determined. Progesterone pretreatment increased IP_3R type 1 single channel open probability at all physiologically relevant Ca^{2+} concentrations but did not affect the overall activity dependence on cytosolic free Ca^{2+} , which can be identified by the preservation of the bell shaped Ca^{2+} dependence curves (figure 4g). However, the IP_3 dependence of the receptors was altered by pre-incubation of mouse rod bipolar cells with progesterone leading to a significant reduction of the EC_{50} (figure 4h). In sum, these data provide evidence for a progesterone-mediated control of neuronal activity and calcium signaling through kinase-mediated signaling thus leading to posttranslational modification and increased gain of the intracellular signaling system controlled by IP_3Rs .

Discussion

We were able to confirm previous reports on the distribution of type 1 IP_3R in rod bipolar cells [13] and to identify it as being co-distributed with the progesterone receptor in the same subcellular regions. This finding allowed us to investigate a potential molecular substrate

for signaling cascades originating from steroid hormone signaling and interfacing with IP₃-mediated Ca²⁺ signaling. Our finding that progesterone-mediated activation of Akt leads to posttranslational modification of IP₃Rs, which in turn results in increased gain of IP₃R mediated Ca²⁺ signaling, is supported by several lines of mechanistic evidence at the cellular and molecular level: The progesterone-mediated Ca²⁺ transient is independent of extracellular Ca²⁺, but is mediated by IP₃-sensitive intracellular stores. Inhibition of PI-3 kinase/Akt/PKB signaling, but not of MAPK abolishes the progesterone-triggered Ca²⁺ signal, phosphorylation of IP₃Rs and potentiation of IP₃R single channel activity. This integration of the direct control of the gain of the Ca²⁺ second messenger system as a signaling gatekeeper for neuronal activity into steroid hormone signaling as identified in the present study defines a novel pathway for interaction of the endocrine and central nervous system.

As reported previously, acute enzymatic and mechanical isolation of rod bipolar cells does not affect cellular morphology and key physiological functional properties [13, 56-61]. Therefore, the present study utilized this model system for the analysis of IP₃R-driven Ca²⁺ signaling and confirmed the maintenance of sub-cellular protein distributions related to this pathway [8, 10-13, 57]. Previous reports that describe the presence of progesterone receptors [57] as well as genomic steroid hormone receptor activity in the retina [80] corroborate our results of progesterone receptor immunoreactivity and function in the present paper. Kobrinsky and colleagues had shown that reduced levels of IP₃R protein decrease progesterone-mediated cellular activity in a model system of oocyte maturation [81]. This study supports our finding that progesterone activity is, at least in part, IP₃R-mediated and is functionally coupled to release of Ca²⁺ from intracellular stores.

One of the potential functional consequences of IP₃R activity potentiated by progesterone is to increase the gain of IP₃Rs. Typically, sub-maximal concentrations of agonist for PLC-coupled neurotransmitter receptors generate low cytosolic IP₃ concentrations and therefore activate IP₃R with high affinity for IP₃ [76, 78, 79, 82, 83] located in the dendrites [13]. Progesterone-mediated increase in IP₃R sensitivity, as seen in the leftward shift in the dose-response curve in figure 4h, would enable IP₃R with a lower affinity for IP₃ [76, 78, 79], expressed predominantly in the soma and the axon terminal of rod bipolar cells

(figure 1 and [13]), to respond to stimuli of lower amplitude. Progesterone could therefore function at the interface between steroid hormone and intracellular Ca²⁺ signaling. This could on one hand set the gain of IP₃R-mediated Ca²⁺ signaling pathways and on the other hand allow progesterone signaling, which has typically been associated with genomic and different sets of cell signaling pathways, access to another critical intracellular signaling pathway. Progesterone could, therefore, influence retinal function through classical genomic mechanisms and/or through activation of other signaling pathways that may also converge on a nuclear endpoint, and at the same time alternatively, and as reported here, modulate IP₃R-mediated intracellular Ca²⁺ signaling.

With respect to overall retinal function, the cytosolic Ca²⁺ concentration of ON-bipolar is critical: photoreceptor cells release the neurotransmitter glutamate which hyperpolarizes ON-bipolar cells including rod bipolar cells through activation of their mGluR6 which inactivates cGMP-gated cation channel on the plasma membrane producing a sign inversion [82]. The function of these cGMP-gated cation channels is modulated by cytosolic Ca²⁺ and with them the adaptation of the ON-pathway to changing light levels within visual neurotransmission [84-86], reviewed in [87]. Data from the present study indicate that steroid hormone signaling has the potential to modulate glutamate signaling through group I mGluR and its effects on the mGluR6 pathway by determining the gain of IP₃R-mediated Ca²⁺ signaling [13, 84-86], reviewed in [87]. This has potential implications for both adaptation to changing light levels as well as control of neuronal Ca²⁺ homeostasis.

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