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ORIGINAL PAPER

Type-1, but Not Type-5, Metabotropic Glutamate Receptors are Coupled to Polyphosphoinositide Hydrolysis in the Retina

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Abstract mGlu1 and mGlu5 metabotropic glutamate receptors are expressed in the vertebrate retina, and are co-localized in some retinal neurons. It is believed that both receptors are coupled to polyphosphoinositide (PI) hydrolysis in the retina and their function may diverge in some cells because of a differential engagement of downstream signaling molecules. Here, we show that it is only the mGlu1 receptor that is coupled to PI hydrolysis in the retina. We used either bovine retinal slices or intact mouse retinas challenged with the mixed mGlu1/5 receptor agonist, DHPG. In both models, DHPG-stimulated PI hydrolysis was abrogated by the selective mGlu1 receptor antagonist, JNJ16259685, but was insensitive to the mGlu5 receptor antagonist, MPEP. In addition, the PI response to DHPG was unchanged in the retina of mGlu5$^{-/-}$ mice but was abolished in the retina of *crv4* mice lacking mGlu1 receptors. Stimulation of the mitogen-activated protein kinase pathway by DHPG in intact mouse retinas were also entirely mediated by mGlu1 receptors. Our data provide the first example of a tissue in which a biochemically detectable PI response is mediated by mGlu1, but not mGlu5, receptors. Hence, bovine retinal slices might be used as a model for the functional screening of mGlu1 receptor ligands. In addition, the mGlu1 receptor caters the potential as a drug target in the experimental treatment of degenerative disorders of the retina.

Keywords mGlu1 receptor · Polyphosphoinositide hydrolysis · Retina · *crv4* mice

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Introduction

Metabotropic glutamate (mGlu) receptors are expressed in all retinal layers, and are potential drug targets in the treatment of retinal disorders [1, 2]. mGlu receptors form a family of eight subtypes, subdivided into three groups on the basis of the amino acid sequence, pharmacological profile and transduction pathways [3]. Group-I includes mGlu1 and mGlu5 receptors, which are coupled to $G_{q/11}$, and are activated by 3,5-dihydroxyphenylglycine (DHPG). This drug, which acts as an orthosteric agonist, is group-selective, but not subtype-selective [4, 5]. Thus, the use of subtype-selective antagonists or the use of mice with genetic deletion of mGlu1 or mGlu5 receptors is necessary to dissect the role of the two subtypes in the biological response to DHPG. Group-II (mGlu2 and mGlu3) and group-III (mGlu4, mGlu6, mGlu7, and mGlu8) receptors are all coupled to G_i/G_o proteins in heterologous expression systems [3].

All mGlu receptor subtypes, with the exception of mGlu3 receptors, modulate synaptic transmission in the vertebrate retina [2]. So far, most of the studies have focused on mGlu6 and mGlu8 receptors, because these two receptors have been linked to autosomal recessive congenital stationary night blindness [6–8] and to an autosomal form of retinite pigmentosa [9], respectively. The knowledge of the role played by retinal mGlu1 and mGlu5

receptors in physiology and pathology is still fragmentary, although these two receptors are particularly attractive as drug targets. Accordingly, mGlu1 and mGlu5 receptors are involved in mechanisms of neurodegeneration and neuroprotection [10], and their expression is enhanced in all retinal layers in response to elevated intraocular pressure in the DBA/2J mouse model for secondary angle-closure glaucoma [11]. mGlu1 and mGlu5 receptors show an overlapping expression in retinal cells. mGlu1α and mGlu5 receptors co-localize in dendrites of ON-bipolar cells [12], and are both present in amacrine cell processes postsynaptic to bipolar cells [12, 13]. mGlu1 receptors are also found in ganglion cells, where the presence of mGlu5 receptors is debated [1]. It has been questioned why two mGlu receptor subtypes sharing (apparently) the same transduction mechanisms are co-localized in retinal cells [12]. Is their function redundant or divergent? And, in the latter case, do mGlu1 and mGlu5 receptors differ in their primary transduction mechanism (polyphosphoinositide (PI) hydrolysis) or in other molecular steps of signal propagation?

Here, we were surprised to find that DHPG-stimulated PI hydrolysis was exclusively mediated by the mGlu1 receptor in the bovine and mouse retina. To our knowledge, there are no other tissues expressing both receptor subtypes in which the mGlu5 receptor does not contribute to a biochemically detectable PI response to DHPG.

Materials and Methods

Materials

(*RS*)-3,5-Dihydroxyphenylglycine (DHPG), 3,4-dihydro-2H-pyrano[2,3-b]quinolin-7-yl-(cis-4-methoxycyclohexyl)-methanone (JNJ16259685), and 2-methyl-6-(phenylethynyl) pyridine (MPEP) were purchased from Tocris Bioscience (Bristol, UK). Myo-[^{3}H]inositol (18 Ci/mmol) was purchased from PerkinElmer (Milan, Italy).

Animals

Eyes and dorsal hippocampi from male young bovines (10–12 months of age) were obtained from a local slaughterhouse. We could not get eyes and brains from bovines of >12 months of age because brain tissue is considered at risk for bovine spongiform encephalopathy. Eyes were enucleated and dorsal hippocampi were dissected within 15 min after death and immediately transferred in ice-cold Krebs-Henseleit buffer (NaCl 118 mM, KCl 4.7 mM, $MgSO_4$ 1.18 mM, KH_2PO_4 1.18 mM, $NaHCO_3$ 24.8 mM, $CaCl_2$ 1.2 mM, D-glucose 10 mM) that had been pre-gassed with 95 % O_2 and 5 % CO_2 to pH 7.4. Tissue was transported to the lab (within 20 min) and used fresh for measurements of PI hydrolysis.

The following strains of mice were used: (i) 2-month old male C57BL/6 J mice (25–30 g, b.w.; Charles River Laboratories, Calco, Italy); (ii) 10–14 weeks old male mGlu5$^{-/-}$ mice and their wild-type counterparts; and (iii) 1-month old male *crv4* mice and their wild-type littermates (provided by A. Puliti). mGlu5$^{+/-}$ mice were originally purchased from Jackson Laboratories (Bar Harbor, ME). The absence of mGlu5 receptors in mGlu5$^{-/-}$ mice was confirmed by Western blot analysis in the cerebral cortex (see below). *crv4* mice are mice of a *BALB*/c/Pas inbred strain carrying a spontaneous recessive mutation that consists of a LTR insertion that disrupts the splicing of the mGlu1-encoding gene, thereby causing the absence of the mGlu1 receptor protein [14]. The lack of mGlu1 receptor protein in *crv4* mice was confirmed by Western blot analysis in the cerebellum. All mice were housed under controlled conditions (temperature, 22 °C; humidity, 40 %) with a 12 h light/dark cycle and food and water ad libitum. All efforts were made to reduce the number of animals used. All experiments were carried out in agreement with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and in compliance with the Italian law on Animal Care No. 26/2014 and the Directive 2010/63/EU. The protocols were approved by the local Animal Care and Use Committee of Neuromed Institute.

Measurement of PI Hydrolysis

Stimulation of the PI hydrolysis was measured in slices prepared from the bovine retina and dorsal hippocampus and in intact mouse retinas, as follows. Bovine retinas and hippocampus were dissected and sliced (350 × 350 μm) with a McIlwain tissue chopper (Mickle Laboratory Engineering, Guildford, UK), and suspended in Krebs/Henseleit buffer (equilibrated with 95 % O_2, 5 % CO_2 to pH 7.4). Slices were incubated in Krebs/Henseleit buffer at 37 °C under constant oxygenation for 30–45 min. Mouse retinas were dissected and directly incubated in oxygenated Krebs/Henseleit buffer for 30–45 min. Gravity-packed slices (40 μl) or intact mouse retinas were transferred to test tubes and incubated for 60 min in 250 μl of Krebs/Henseleit buffer containing 1 μCi of myo-[^{3}H]inositol. At the end of this incubation, LiCl (10 mM) was added, and, after 15 min, slices were challenged with the mGlu1/5 receptor agonist, DHPG, and JNJ16259685 or MPEP, if present, were added 5 min prior to DHPG. After 60 min of incubation, reaction was terminated by addition of methanol:chloroform (2:1, 900 μl), after washing the slices with ice-cold buffer. After further addition of 300 μl chloroform and 600 μl water, samples were centrifuged at low speed to facilitate phase separation, and the upper

aqueous phase was loaded into columns containing 1 ml of Dowex1-X-8 resin (100–200 mesh, formate form; Dow Chemical Company, Midland, MI). [^{3}H]Inositol-monophosphate (InsP) was separated using formate salts of increasing concentrations as a mobile phase. Columns were washed twice with water, once with a solution of 5 mM sodium tetraborate and 40 mM sodium formate to elute cyclic InsP and glycerophosphoinositols, and then with 6.5 ml of 0.2 M ammonium formate and 0.1 M formic acid for the elution of InsP [15]. The remaining aqueous phase and the organic phase were dried under a continuous nitrogen stream, and 0.5 N NaOH was added to each sample. Proteins were measured as described by Lowry et al. [16].

Western blot analysis in brain tissues

Western blot analysis of mGlu1 and mGlu5 receptors was performed in the cerebellum of *crv4* mice and in the cerebral cortex of mGlu5$^{-/-}$ mice (and their wild-type counterparts), respectively. The tissues were homogenized and lysed on ice with RIPA buffer containing protease inhibitors cocktail (Calbiochem, Merck Millipore, Italy) for 30 min and an aliquot was used for protein determination. Lysates were separated on 8 % SDS-PAGE at 100 V for 1 h and transferred on Immuno PVDF membranes (Bio-Rad, Milan, Italy) for 7 min using Trans Blot Turbo System (Bio-Rad, Milan, Italy). The membrane was incubated overnight in blocking buffer (TBS, 0.05 % Tween-20 and 5 % nonfat milk) and then for 2 h with mouse monoclonal anti-mGlu1 antibody (1:700, BD Biosciences, Milan, Italy) or rabbit monoclonal anti-mGlu5 antibody (1:2500, Abcam, Cambridge, UK). Filters were washed three times in Tris-Tween buffered saline and then incubated for 1 h with secondary peroxidase-coupled antibody (anti-rabbit 1:7000 and anti-mouse 1:7000, Calbiochem, Milan, Italy). Immunostaining was revealed by enhanced chemiluminescence luminosity (Amersham, GE Healthcare Life Science, Milan, Italy). The blots were re-probed with monoclonal anti-β-actin antibody 1:50,000 (Sigma).

Study of the Mitogen-Activated Protein Kinase (MAPK) and Phosphatidylinositol-3-Kinase (PI3K) Pathways in Intact Mouse Retinas

Mouse retinas from C57BL/6J were incubated in oxygenated Krebs/Henseleit buffer at 37 °C for 20 min, and then challenged with DHPG for 15 min. If present, JNJ16259685 and MPEP were added 2 min prior to DHPG. The reaction was stopped on ice with lyses buffer (50 mM Tris–HCl, 1 % Nonidet P-40, 0.1 % SDS, 150 mM NaCl, 2 mM EDTA, 50 mM NaF) containing a cocktail of protease inhibitors (Calbiochem, Merck Millipore, Italy) and phosphatase inhibitors (Roche, Life Science, Italy) for 30 min, and an aliquot was used for protein determination. Lysates were separated on 8 % SDS-PAGE at 100 V for 1 h and transferred on Immuno PVDF membranes (Bio-Rad, Milan, Italy) for 7 min using the Trans Blot Turbo System (Bio-Rad, Milan, Italy). Membranes were incubated for 2 h in blocking buffer (TBS, 0.05 % Tween-20, 1 % nonfat milk and 1 or 5 % BSA for p-ERK1/2 and p-Akt, respectively) and then incubated overnight with a mouse monoclonal anti-phosphorylated extracellular signal-regulated kinase (p-ERK1/2) antibody (1:1000, Santa Cruz Biotechnology Inc., Heidelberg, Germany), or a rabbit polyclonal anti-phosphorylated Akt (Ser473) antibody (1:1000; Cell Signaling Technology Inc., Danvers, MA). Filters were washed three times in Tris-Tween buffered saline and then incubated for 1 h with secondary anti-mouse or anti-rabbit peroxidase-coupled antibodies (1:7000; Calbiochem, Milan, Italy). Immunostaining was revealed by enhanced chemiluminescence luminosity (Amersham, GE Healthcare Life Science, Milan, Italy). Data were normalized for the expression of rabbit polyclonal unphosphorylated ERK1/2 or Akt (1:3000, Cell Signaling).

Statistical Analysis

Statistical analysis was performed by One-Way ANOVA followed by the Newman–Keuls or Fisher's LSD post hoc tests to isolate the differences. All data are presented as mean ± SEM.

Results

Measurements of DHPG-Stimulated [^{3}H]InsP Formation in the Bovine Retina

Experiments were carried out using retinas and dorsal hippocampi from male young bovines of 10–12 months of age. In spite of the time elapsed from the death of the animals and the beginning of the incubation (approximately 40–45 min), retinal slices showed a twofold increase in [^{3}H]InsP formation in response to the mixed mGlu1/5 receptor agonist, DHPG. DHPG was used at concentrations of 200 μM, which are known to produce a near-to-maximal PI response in rodent brain slices [17]. DHPG was combined with either MPEP or JNJ16259685, which act as negative allosteric modulators (NAMs) of mGlu5 and mGlu1 receptors, respectively [18, 19]. DHPG-stimulated [^{3}H]InsP formation remained unchanged in the presence of MPEP (3 μM, applied 5 min prior to DHPG), but was abrogated by JNJ16259685 (10 μM). Neither

MPEP nor JNJ16259685 had any detectable effect on PI hydrolysis on their own (Fig. 1a). To exclude that this unexpected pharmacological profile was a general prerogative of bovine tissue, we extended the analysis to bovine hippocampal slices, where DHPG stimulated [^{3}H]InsP formation to a greater extent than that observed in retinal slices. In hippocampal slices, however, MPEP was able to antagonize DHPG-stimulated PI hydrolysis, and was even more effective than JNJ16259685 (Fig. 1b).

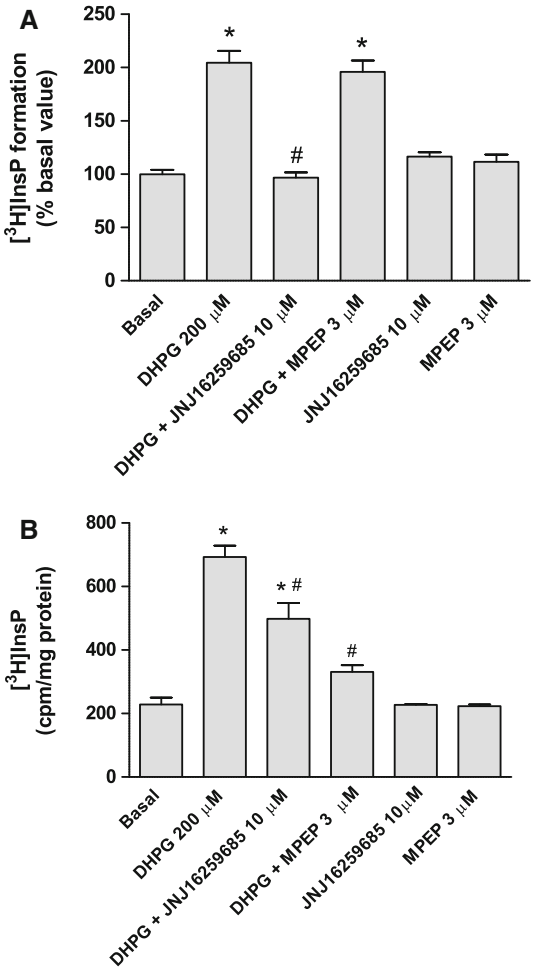

Fig. 1 DHPG-stimulated PI hydrolysis is mediated by the activation of mGlu1 receptors in the bovine retina. DHPG-stimulated PI hydrolysis in slices prepared from bovine retinas is shown in (**a**), where data are expressed as percent of basal and were calculated from 3 independent experiments in which $n = 2$–5 for each treatment in each individual experiment. Values are mean ± SEM. $p < 0.05$ (One-way ANOVA + Newman-Keuls test) versus basal values (*) or versus values obtained after addition of DHPG alone (#). $F_{(5,52)} = 30.28$. Basal values (expressed as c.p.m. of [^{3}H]InsP/mg protein) in the 3 experiments are the following: 304 ± 15; 286 ± 22; 486 ± 61; respectively). DHPG-stimulated PI hydrolysis in bovine hippocampal slices is shown in (**b**), where values are mean ± SEM of 4 determinations from a single experiment. $p < 0.05$ (One-way ANOVA + Newman–Keuls test) versus basal values (*) or versus values obtained after addition of DHPG alone (#). $F_{(5,14)} = 32.75$

Measurements of DHPG-Stimulated [^{3}H]InsP Formation in the Mouse Retina

Because the amount of tissue was not sufficient for slice preparation, we measured DHPG- stimulated PI hydrolysis in intact mouse retinas (see "Materials and Methods" section). Although the use of intact tissue may have limited the diffusion of DHPG to internal retinal layers, we were able to obtain a substantial PI response to DHPG in our assay. In the mouse retina, DHPG stimulated [^{3}H]InsP formation to a greater extent than in bovine retinal slices (2.5 vs. twofold).

The PI response to DHPG in the mouse retina was insensitive to MPEP and abolished by JNJ16259685 (Fig. 2a), in nice agreement with data obtained in bovine retinal slices. We could detect both the mGlu1 and mGlu5 receptor protein by immunoblot analysis in the mouse retina (Fig. 2b, c), although the amount of mGlu1 receptor protein was much lower than that detected in the mouse cerebellum (in Fig. 2b, 60 μg of retinal proteins and 10 μg of cerebellar proteins were loaded to the gel). These data are in line with immunohistochemical and electron microscopic findings showing the presence of mGlu1 and mGlu5 receptors in the rodent and chicken retinas [11, 12, 20].

DHPG-Stimulated PI Hydrolysis in the Retinas of Mice with Genetic Deletion of Either mGlu1 or mGlu5 Receptors

We extended the analysis to the retinas of either mGlu5$^{-/-}$ mice or *crv4* mice and their wild-type counterparts.

DHPG stimulated [^{3}H]InsP formation to the same extent in mGlu5$^{-/-}$ mice and their wild-type counterparts (Fig. 3a), confirming that the mGlu5 receptor does not contribute to the PI response to DHPG in the retina. The lack of mGlu5 receptors in all mGlu5$^{-/-}$ mice used for measurements of DHPG-stimulated PI hydrolysis in the retina was confirmed by immunoblot analysis of the mGlu5 receptor protein in the cerebral cortex (Fig. 3b).

We also used *crv4* mice that lack the mGlu1 receptor protein because of a spontaneous recessive mutation of the *Grm1* gene [14]. In wild-type littermates, DHPG enhanced [^{3}H]InsP formation to a greater extent than usually observed in the mouse retina (compare Fig. 4a to Fig. 2a), perhaps because younger mice (1-month of age) were used in this particular experiment. DHPG-stimulated PI hydrolysis was nearly abolished in the retinas of *crv4* mice (Fig. 4a). The lack of mGlu1 receptors in all *crv4* mice used for measurements of PI hydrolysis in the retina was confirmed by immunoblot analysis of the mGlu1 receptor protein in the cerebellum (Fig. 4b).

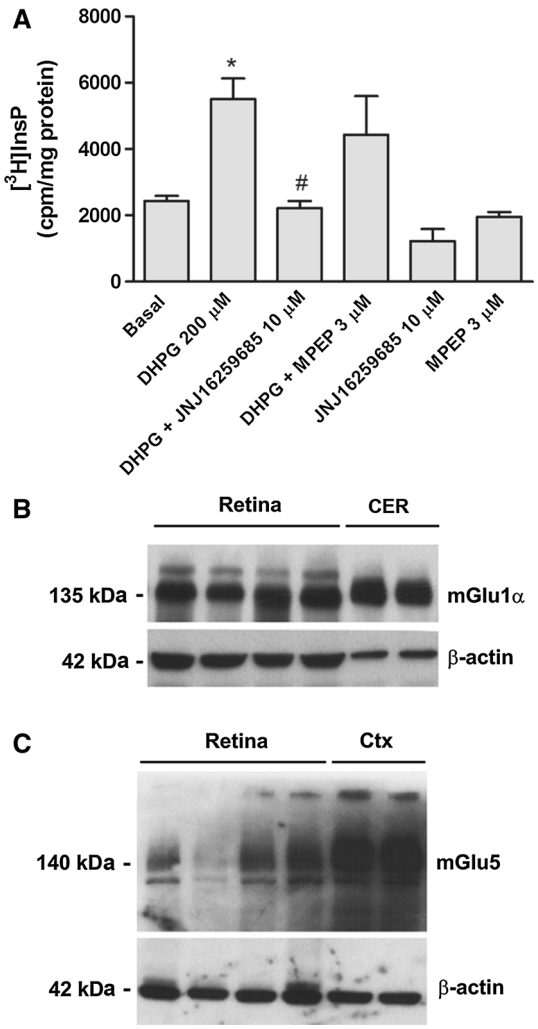

Fig. 2 DHPG-stimulated PI hydrolysis is sensitive to mGlu1 receptor blockade, but not to mGlu5 receptor blockade, in the mouse retina. DHPG-stimulated PI hydrolysis in intact mouse retinas is shown in (**a**), where values are mean ± SEM of 3–5 determinations. $p < 0.05$ (One-way ANOVA + Newman–Keuls test) versus basal values (*) or versus values obtained after addition of DHPG alone (#). $F_{(5,18)} = 7.69$. Western blot analysis of mGlu1 and mGlu5 receptors in the retinas of C57BL/6J mice is shown in (**b**) and (**c**), respectively. In (**b**), the band at 135 kDa corresponds to the molecular weight of the mGlu1α receptor monomer. In (**c**), the band at 140 kDa corresponds to the mGlu5 receptor monomer. The higher molecular size band likely corresponds to the mGlu5 receptor dimer. In (**b**), 60 μg of retinal proteins and 10 μg of cerebellar protein were loaded on each *lane*. In (**c**), 30 μg of retina and cortical proteins were loaded on each *lane*. *CER* cerebellum; *Ctx* cerebral cortex

Study of Other Signaling Pathways Activated by Group-I mGlu Receptor Activation

We extended the study to the MAPK and PI3K pathways, which are known to be activated in response to group-I mGlu receptor stimulation [3]. Addition to DHPG (200 μM) to intact mouse retinas increased p-ERK1/2 levels by more than two fold after 15 min of incubation (Fig. 5). The response to DHPG was inhibited by JNJ16259685 (10 μM) but not by MPEP (3 μM), as already observed for DHPG-stimulated PI hydrolysis (Fig. 5). Neither JNJ16259685 nor MPEP caused changes in p-ERK1/2 levels when applied in the absence of DHPG (Fig. 5). At least under our experimental conditions, we were unable to find any stimulation of the PI3K pathway by DHPG in intact mouse retinas (not shown).

Discussion

Immunohistochemical and electron microscopic studies have shown that both mGlu1α and mGlu5 receptors are present in dendrites of ON-bipolar cells of rodent and chicken retinas [10, 12, 20]. The effector systems activated by mGlu1 and mGlu5 receptors in ON bipolar cells are unknown. mGlu1 and mGlu5 receptors have been studied in greater detail in retinal amacrine cells, which are inhibitory GABAergic neurons forming reciprocal synapses with bipolar cells. Interestingly, a large body of evidence indicates that mGlu1 and mGlu5 receptors display opposite functions in amacrine cells. In these cells, activation of mGlu1 receptors enhances GABA release, reduces the activity of L-type voltage-sensitive Ca^{2+} channels (VSCCs), and reduces GABA responses as a result of calcineurin-dependent $GABA_{A/C}$-receptor dephosphorylation. In contrast, activation of mGlu5 receptors enhances the activity of L-type VSCCs and enhances GABA responses as a result of protein kinase C (PKC)-dependent phosphorylation of $GABA_A$ receptors [2, 21–25]. In ganglion cells, non-subtype selective drugs that activate both mGlu1 and Glu5 receptors modulate the activity of L-type and N-type VSCCs [26, 27]. The relative contribution of mGlu1 and mGlu5 receptors to this effect is unknown. Of note, the mGlu5 receptor is expressed and functional in retinal glial Muller cells. Activation of mGlu5 receptors suppresses Kir4.1 potassium currents and enhances the expression of glial fibrillary acidic protein (an indicator of reactive gliosis) in a rat model of chronic ocular hypertension [28].

In recombinant cells, both mGlu1 and mGlu5 receptors stimulate PI hydrolysis with ensuing formation of $InsP_3$ and diacylglycerol (DAG) [3]. It has been assumed that both mGlu1 and mGlu5 receptors are coupled to PI hydrolysis in retinal cells, and that the different functions of the two receptors in the same cell type (e.g. amacrine cells), reflect the involvement of different scaffolding proteins, a different kinetics of intracellular Ca^{2+} mobilization [29], or the engagement of different molecular processes that lies downstream of PI hydrolysis. Our data show that this is not the case. Unexpectedly, the PI response to the mixed mGlu1/5 receptor agonist, DHPG, in

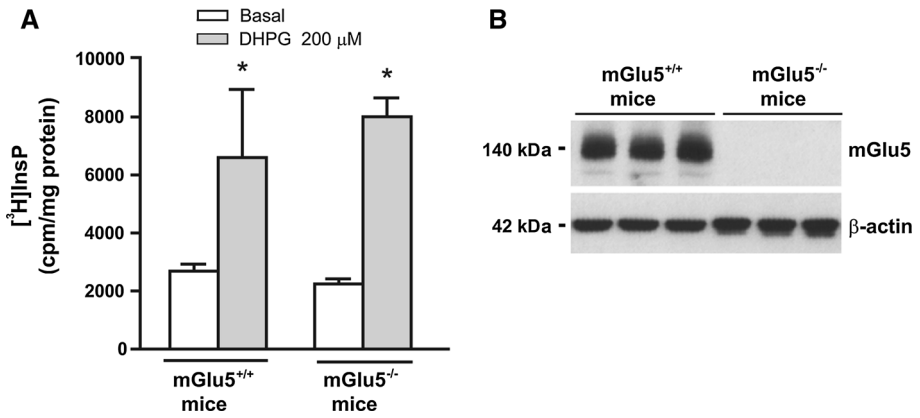

Fig. 3 DHPG-stimulated PI hydrolysis is unchanged in retinas from mGlu5 knockout mice. Stimulation of PI hydrolysis by DHPG in intact retinas from mGlu5$^{+/+}$ and mGlu5$^{-/-}$ mice is shown in (**a**), where values are mean ± SEM of 3 determinations. *$p < 0.05$ (One-way ANOVA + Newman–Keuls test) versus the respective basal values. $F_{(3,8)} = 5.41$. Western blot analysis of mGlu5 receptors in the cerebral cortex of all mGlu5$^{+/+}$ and mGlu5$^{-/-}$ mice used for measurements of PI hydrolysis in the retina is shown in (**b**)

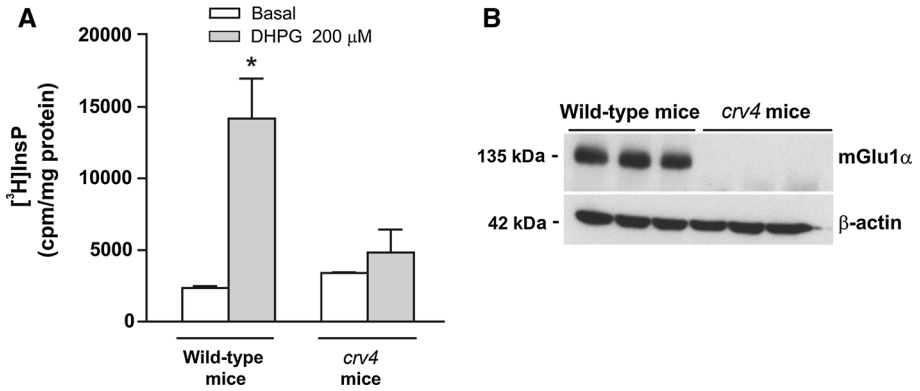

Fig. 4 DHPG-stimulated PI hydrolysis is abrogated in retinas from *crv4* mice lacking mGlu1 receptors. Stimulation of PI hydrolysis by DHPG in intact retinas from wild-type and *crv4* mice is shown in (**a**), where values are mean ± SEM of 3 determinations. *$p < 0.05$ (One-way ANOVA + Newman–Keuls test) versus the respective basal values. $F_{(3,7)} = 9.59$. Western blot analysis of mGlu1 receptors in the cerebellum of all wild-type and *crv4* mice used for measurements of PI hydrolysis in the retina is shown in (**b**)

the bovine or mouse retina was entirely mediated by mGlu1 receptors with no apparent contribution of mGlu5 receptors. We wish to highlight that our biochemical assay is based on the ratio between a signal generated by activation of group-I mGlu receptors and a noise (basal InsP formation) that reflects the constitutive turnover rate of membrane inositol phospholipids and the (possible) endogenous activation of multiple receptors coupled to PI hydrolysis. We cannot exclude that the noise here was too high to detect small increases in [^{3}H]InsP formation caused by mGlu5 receptor activation. Accordingly, the effect of mGlu5 receptor activation of Kir current in glial Muller cells, at least under pathological conditions, appears to be mediated by the stimulation of PI hydrolysis [28]. In spite of this potential bias (which is inherent to the analytical method), we are not aware of studies carried out on brain tissue in which the PI response to DHPG is insensitive to mGlu5 receptor blockade and is entirely mediated by mGlu1 receptors.

We first examined the pharmacological profile of the PI response in the bovine retina, which offers the advantage of the large amount of tissue, but harbors the disadvantage of the absence of any information on the expression of mGlu1 and mGlu5 receptors. We could not examine the expression of mGlu1 or mGlu5 receptors in the bovine retina because our mGlu1 and mGlu5 antibodies did not label the respective receptors in bovine tissue (not shown). However, it was interesting to observe that the PI response to DHPG in bovine retinal slices was abrogated by pharmacological blockade of mGlu1 receptors but was instead insensitive to mGlu5 receptor blockade. This pharmacological profile was peculiar to the retina because the mGlu5 NAM, MPEP, was highly effective in reducing the PI response to DHPG in the bovine hippocampus.

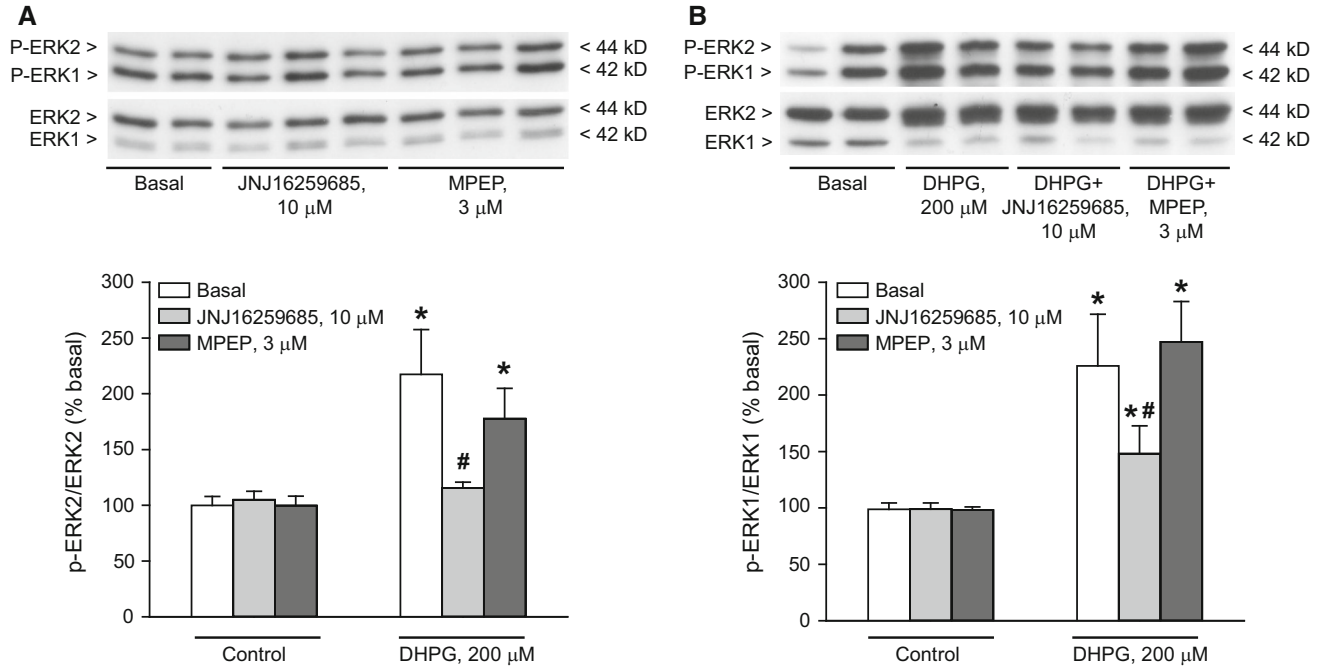

Fig. 5 DHPG-stimulated MAPK activation is mediated by mGlu1 receptors in the mouse retina. Levels of p-ERK1 and p-ERK2 in intact mouse retinas incubated with DHPG in the absence or presence of JNJ16259685 or MPEP are shown in (**a**) and (**b**), respectively. Representative immunoblots are shown on the *top*. Values are expressed as percentage of the respective basal values, and are mean ± SEM of 6–10 determinations from 5 different immunoblots. $p < 0.05$ (One-way ANOVA ± Fisher LSD) versus the respective controls (*) or versus the respective basal values (i.e. DHPG alone) (#). $F_{(5,39)} = 7.91$ and 9.82 in (**a**) and (**b**), respectively

The analysis of the PI response in the mouse retina required the incubation of a single intact retina per test tube, which offered the advantage of a more reliable statistical analysis, but the potential disadvantage of a limited diffusion of DHPG (a hydrophilic compound) in the tissue. We could detect a substantial and highly reproducible PI response to DHPG in the mouse retina, the extent of which was similar to that reported with the group-I mGlu receptor agonist, quisqualate, in rabbit retinal slices [30]. Again, stimulation of PI hydrolysis by DHPG in intact mouse retinas was abrogated by JNJ16259685 and totally insensitive to MPEP. The inability of the mGlu5 receptors to activate PI hydrolysis in the retina was confirmed using mGlu5$^{-/-}$ mice, in which the PI response to DHPG remained unchanged. We also used mice carrying the spontaneous recessive mutation cervelet-4 (*crv4* mice) in the gene encoding for mGlu1 receptors. These mice lack functional mGlu1 receptors, and are phenotypically characterized by severe ataxia and kidney damage [14, 31]. DHPG failed to stimulate [^{3}H]InsP formation in the retinas of *crv4* mice, confirming that the PI response to DHPG in the retina is entirely mediated by mGlu1 receptors. It will be interesting to examine whether *crv4* mice or mGlu1$^{-/-}$ mice show abnormalities in retinal function during development and in the adult life. To our knowledge, there are no studies on visual pathways in mice lacking mGlu1 receptors.

In conclusion, we have demonstrated for the first time that only a PI response mediated by mGlu1 receptors, and not a response mediated by mGlu5 receptors, can be detected in intact retinas or in retinal slices. We also examined whether activation of other signaling pathways controlled by group-I mGlu receptors [3] could be mediated by mGlu5 receptors in the retina. Under our experimental conditions activation of the MAPK pathway, but not activation of the PI3K pathway, by DHPG could be detected in intact mouse retinas. Interestingly, also this transduction pathway appeared to be entirely mediated by mGlu1 receptors. Whether other pathways are specifically activated by mGlu5 receptors in the retina remains to be determined.

Because the increase in intracellular Ca^{2+} that follows PI hydrolysis has been implicated in mechanisms of group-I mGlu receptor-mediated neurodegeneration [10], our findings pay the way to the experimental use of mGlu1 receptor in the treatment of pathological conditions that cause degeneration of retinal neurons, such as macular degeneration and glaucoma. It will be interesting to examine whether chronic treatment with JNJ16259685 or other mGlu1 receptor NAMs in DBA/2J mice exerts a protective activity against retinal degeneration caused by an elevated intraocular pressure, and whether neuroprotection occurs at doses that cause only limited cerebellar adverse effects [32]. The evidence that mGlu1 receptors are up-regulated in all retinal

layers of DBA/2 J mice in response to elevated ocular pressure strongly encourages the experimental use of mGlu1 receptor NAMs in these mice. However, as a word of caution, we cannot exclude that the mGlu5 receptor has a role in mechanisms of neurodegeneration/neuroprotection associated with retinal disorders, keeping in mind that mGlu5 receptors participate to mechanisms of reactive gliosis in the retina [28] (see above). From the perspective of drug development, the selectivity of mGlu1 receptors in mediating the PI response in the retina offers the unique opportunity to screen compounds for a specific activity on mGlu1 receptors in a native environment. In particular, the large amount of slices obtained from bovine retinas allows a near-to-complete analysis of novel mGlu1 compounds on PI hydrolysis without contaminating effects mediated by mGlu5 receptors.

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