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Glutamate Accelerates RPE Cell Proliferation Through ERK1/2 Activation Via Distinct Receptor-Specific Mechanisms

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Abstract The proliferation and migration of Retinal Pigment Epithelium cells resulting from an epithelial-mesenchymal transition plays a key role in proliferative vitreoretinopathy, which leads to retinal detachment and the loss of vision. In neurons, glutamate has been shown to activate the Ras/Raf/MEK/ERK cascade, which participates in the regulation of proliferation, differentiation, and survival processes. Although glutamate-stimulation and the activation of ERK1/2 by different stimuli have been shown to promote RPE cell proliferation, the signaling pathway(s) linking these effects has not been established. We analyzed the molecular mechanisms leading to glutamate-induced proliferation by determining ERK1/2 and CREB phoshporylation in chick RPE cells in primary culture and the human-derived RPE cell line ARPE-19. This study shows for the first time, that glutamate promotes RPE cell proliferation by activating two distinct signaling pathways linked to selective glutamate receptor subtypes. Results demonstrate that glutamate stimulates RPE cell proliferation as well as ERK and CREB phosphorylation. These effects were mimicked by the mGluR agonist ACPD and by NMDA, and were prevented by the respective receptor inhibitors MCPG and MK-801, indicating a cause-effect relationship between these processes. Whereas mGluR promoted proliferation by activating the MEK/ERK/CREB cascade, NMDA stimulated proliferation through the MEK-independent activation of Ca^{2+}/calmodulin-dependent kinases. The blockage of both signaling pathways to proliferation by KN-62 suggests the involvement of CaMKs in the control of glutamate-induced proliferation at a common step, downstream of CREB, possibly the regulation of cell cycle progression. Based on these findings, the participation of glutamate in the development of PVR can be considered. J. Cell. Biochem. 104: 377–390, 2008. © 2007 Wiley-Liss, Inc.

Key words: cell proliferation; GluRs; ERK1/2; CaMKs; cell cycle

The retinal pigment epithelium (RPE) plays an essential role in the function and maintenance of the neural retina [Strauss, 2005]. Adult retinal pigment epithelial cells are quiescent, differentiated, and reside in the G_o phase of the cell cycle however, under pathological conditions involving the alteration of the Blood-Retina Barrier (BRB), RPE cells are exposed to a variety of growth factors, cytokines, and neurotransmitter compounds contained in serum [Limb et al., 1991; Abe et al., 1996]. RPE cell activation results in proliferation, which is a central phenomenon in the pathogenesis of proliferative retinal diseases, such as proliferative vitreoretinopathy (PVR), a major cause of retinal surgery failure [Ando et al., 2000; Pastor et al., 2002]. PVR is characterized by the epithelial-mesenchymal transition of the RPE cells ("fibroblastic transformation"), from mitotically inactive epithelial cells, to actively dividing fibroblast-like cells with the ability to migrate [Grisanti and Guidry, 1995; Casaroli-Marano et al., 1999]. These alterations result in the formation of contractile epiretinal membranes in the vitreous cavity and both surfaces of the retina, mainly composed by transformed RPE cells and glial cells; the contraction of these membranes, eventually causes retinal detachment and the loss of vision [Ando et al., 2000].

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Glutamate, the main excitatory neurotransmitter in the radial signaling pathway of the vertebrate retina [Massey and Miller, 1990] has been shown to regulate the proliferation, migration, and survival of neuronal progenitors and immature neurons [Komuro and Rakic, 1993; Guerrini et al., 1995], and also to play a key role in synaptic plasticity and gene expression, as well as in excitotoxic neuronal death [Thomas and Huganir, 2004; Wang et al., 2007]. Glutamate has been shown to promote RPE cell proliferation indirectly, through an FGF-mediated mechanism [Uchida et al., 1998; Bryckaert et al., 2000]. Additionally, the subunit-expression and pharmacology of glutamate receptors in human trans-differentiated RPE cells differ significantly from those in normal quiescent cells [López-Colomé et al., 1994]. Since the functional properties of GluRs depend on the differential expression and post-transcriptional processing of receptor subunits, such differences could contribute to the alteration of morphology and cell division rate observed in PVR. However, glutamate receptor specific signaling in RPE and its possible involvement in the control of proliferation has not been studied.

Glutamate actions are accomplished through the stimulation of ionotropic receptors (iGluRs) and G protein-coupled metabotropic receptors (mGluRs). The superfamily of iGluRs includes the NMDA, AMPA and KA receptor families, Na^{+}- or Ca^{++}-permeable cation channels, whereas eight mGluRs, linked to the activation of second messenger cascades have been identified, and classified in groups I, II, and III according to their pharmacology and sequence homology [Michaelis, 1998].

Numerous studies have shown that the stimulation of iGluRs and mGluRs in neuronal and non-neuronal cells triggers the activation of the mitogen-activated protein kinase (MAPK) pathway [Mao et al., 2004; Thomas and Huganir, 2004; Haddad, 2005; Wang et al., 2007]. MAPKs, particularly ERK 1/2, play an important role in proliferation, differentiation and survival processes, as well as in pathological cell migration [López-Colomé and Ortega, 1997; Fukunaga and Miyamoto, 1998; Pearson et al., 2001; Wang et al., 2004; Xu and Deng, 2006].

The Ras-MAPK cascade involves the sequential activation of four signaling proteins: the GTP-binding protein Ras, and the kinases Raf, MEK, and ERK. Receptor and non-receptor tyrosine kinases, PKC- and Ca^{++}/calmodulin-dependent pathways have been shown to regulate this cascade, through the activation of the small GTPase Ras, the first step in this signaling mechanism [Yoon and Seger, 2006]. Upon the Ras-mediated activation of Raf, which activates the dual function kinase MEK, ERK1/2 phosphorylation on Tyr 204 and Thr 202 by this kinase, leads to the activation of nuclear and cytoplasmic ERK-substrates [Roux and Blenis, 2004]. This process has been shown to play an important role in the control of cell proliferation by the ERK-induced activation of transcription factors NF-κB, c-Myc, CREB, and AP-1 [Chang et al., 2003], as well as by promoting the expression of regulatory proteins involved in the cell cycle. ERK activation increases the activity of CDKs, such as CDK2, and has also been shown to induce the expression of cyclin D1 via Fos family members, and to regulate the assembly of cyclin E/CDK2 complex [Musgrove, 2006; Chambard et al., 2007]. Also on this line, the activation of $p90^{RSK}$ by ERK1/2 is involved in the regulation of cell cycle through the phosphorylation of substrates such as CREB and Fos [Frödin and Gammeltoft, 1999; Shaulian and Karin, 2002], as well as by the activation of MAPK-interacting kinase MNK1 which in turn phosphorylates the translation initiation factor 4E (eIF4E) [Chambard et al., 2007].

Downstream of ERK1/2, the phosphorylation of cAMP response element-binding protein (CREB) by several stimuli such as growth factors and neurotransmitters turns on the transcription of more than 5,000 target genes, including those coding for cell cycle regulatory proteins such as cyclin D2 and cyclin A1, which regulate G1 to S cell cycle phase transition by activating cyclin-dependent kinase 2 [Siu and Jin, 2007].

Cell proliferation in PVR seems to involve multiple factors [Kaven et al., 2000]. Among these, it is conceivable that RPE cell proliferation and/or epithelial-mesenchymal transition, pivotal processes in the development of PVR, could be directly induced by glutamate through the activation of ERK1/2, the phosphorylation of transcription factors such as CREB and Fos [Vanhoutte et al., 1999], and the eventual modification of gene expression. This assumption is supported by findings showing that high concentrations of NMDA induce CREB phosphorylation and

DNA-binding activity of CRE in the retina [Isenoumi et al., 2004], as well as by a more recent study which demonstrates that the specific activation of NMDA receptors stimulates cancer cell division due to the activation of ERK1/2 signaling [Stepulak et al., 2005].

The present study was aimed to analyzing the effect of glutamate on the activation of the ERK1/2 cascade as a possible mechanism involved in the induction of RPE proliferation, using chick RPE cells in primary culture and human-derived ARPE-19 cell line as model systems.

MATERIALS AND METHODS

Cell Culture

Primary culture of RPE cells was carried out as previously described [López-Colomé et al., 1993]. Eyes from 7-day-old chick embryos were enucleated, the lens and the retina were discarded, and the eye-cups were placed in Krebs-Ringer Bicarbonate buffer (KRB) pH 7.4 containing (in mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.17; KH_2PO_4, 2; $NaHCO_3$, 25; glucose, 5.6, and rinsed four times. The RPE was dissected and dissociated in Opti-Mem medium (GibcoBRL) following 5 min incubation in phosphate buffered saline, pH 7.4, containing 0.13% trypsin. The cells were plated onto 3.5-cm-diameter multi-well dishes, at a density of 1.2×10^5 cells/well, and incubated at 37°C in Opti-Mem supplemented with 4% fetal bovine serum (FBS), 0.25% Penicillin-Streptomycin-Neomycin, and 0.1% Nistatin. All assays were performed in 5-day-old cultures. Cell morphology was assessed by optic microscopy. The human-derived RPE cell line ARPE-19 (ATCC) was propagated in D-MEM/F12 medium (ATCC) containing 4% FBS, as described previously [Dunn et al., 1996].

Cell-Proliferation

RPE cell proliferation was measured by [^{3}H]-thymidine incorporation. Cells were seeded and maintained for 24 h in medium containing 4% FBS. The medium was then replaced by medium containing 1% FBS in the absence (control), or the presence of glutamate receptor agonists: 1 mM L-glutamic acid; 150 μM amino-cyclopentane-1, 3-dicarboxylic acid (ACPD), α-amino-3-hydroxy-4-isoxazolepropionic acid (AMPA) or N-Methyl-D-aspartic acid (NMDA) (Tocris). When tested, glutamate receptor antagonists α-Methyl-4-carboxyphenylglycine (MCPG; 50 μM); 6-Cyano-7-nitroquinoxaline-2, 3-dione (CNQX; 10 μM); dizocilpine maleate (MK-801; 10 μM), and 10 μM of the CaMK inhibitors KN-62 or KN-93 (Tocris) were included 3 h previous to the addition of the agonist. After 24 h, 0.5 μCi/ml [^{3}H]-thymidine (PerkinElmer; 16 Ci/mmol) was added, and cultures were further incubated for 16 h. Following this period, cultures were rinsed twice in PBS and incubated for 1 h with 10% trichloroacetic acid, solubilized in 0.5% sodium dodecyl sulfate (SDS), and counted for radioactivity in a liquid scintillation counter (Beckman).

ERK-Stimulation Protocol

Following 4 days in culture in the presence of 4% FBS, cells were serum-deprived for 12 h. One millimolar Glutamate or 150 μM glutamate receptor agonists were then included for 10 min. When tested, glutamate receptor antagonists, the MEK inhibitor PD98059 (30 μM), and the CaMK inhibitors KN-62 and KN-93 (10 μM) as well as the inactive KN-93 analog, KN-92, were included in the medium 3 h prior to the addition of the agonists. At the end of the stimulation period, cells were lysed in 50 mM Tris–HCl buffer pH 7.4, containing 150 mM NaCl, 10 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% Chaps, 0.5% NP40, 0.1% BSA, 10% protease inhibitor cocktail, 40 mM β-glycerophosphate, and 10 mM sodium pyrophosphate.

Immunoprecipitation

The soluble fraction of the lysates was incubated with sepharose-coupled protein-A at 4°C for 1 h. The Protein-A beads were pelleted, and the supernatant was preserved. The beads were incubated at 4°C for 1 h with anti-ERK1/2 polyclonal antibody (Calbiochem; 10 μg/ml), and the immune complex formed was subsequently added to the Protein-A-bead-supernatant and incubated at 4°C for 3 h. Following this period, the samples were washed with lysis buffer and used in ERK activation assays.

ERK1/2 Activation Assay

The ERK immunoprecipitate was washed in Hepes 20 mM, pH 8.0, containing 10 mM $MgCl_2$,

1 mM DTT, 0.5 mM EGTA, and 40 μM ATP. Samples were added 5 μCi of [γ^{32}P]-ATP (PerkinElmer Life Science; 6000 Ci/mmol), incubated at 37°C for 10 min, washed three times in buffer containing 40 μM unlabeled ATP, resuspended in Laemmli buffer containing: 0.75 M Tris–HCl pH 8.8, 5% SDS, 20% glycerol, 0.01 bromophenol blue, and boiled for 5 min.

Western Blotting

The samples were resolved by polyacrylamide gel electrophoresis (10%), under denaturing conditions (SDS–PAGE), and electro-transferred onto polyvinyldiene difluoride (PVDF) membranes (Amersham Biosciences). After autoradiography, the membranes were incubated in blocking buffer (5% Milk, 2% BSA in Tween TBS) at room temperature for 1 h, followed by further incubation for 1 h in the presence of anti-ERK1/2, 1:5,000. The membranes were washed in Tween TBS, added anti-rabbit horseradish peroxidase (HRP) antibody (Zymed Laboratories, Inc.) in the same solution, and developed using an enhanced chemiluminscence kit (ECL; Amersham Biosciences). The density of immunoblotted bands was determined using Quantity One 1-D Analysis Software (Bio-Rad), and normalized to control values. The same protocol was used for anti-p-CREB (Cell Signaling) detection.

RESULTS

The Activation of Ionotropic and Metabotropic Glutamate Receptors Enhances RPE Cell Proliferation

We tested the effect of glutamate on the proliferation of RPE cells in culture, using [^{3}H]-thymidine incorporation. Our results show a 100% increase in the proliferation of RPE cells maintained in the presence of 1 mM glutamate for 36 h, compared to control cultures. Because chick RPE cells necessarily require serum for survival, proliferation was assessed in the presence of low (1%) FBS (Fig. 1A). In order to rule-out a contribution of serum-contained factors to the observed increase in proliferation, identical experiments were performed in the human-derived RPE cell line ARPE-19 in the complete absence of serum (SFM), since these cells are viable in such condition. The specific glutamate receptor subtypes involved in glutamate-induced proliferation were identified by analyzing the effect of the general Group-I mGluR agonist ACPD, and the ionotropic GluR agonists AMPA and NMDA on proliferation. Results in Figure 1A show that ACPD and NMDA significantly stimulated cell proliferation, whereas AMPA was ineffective. The same experimental protocol was applied to cultures of the human RPE cell line ARPE-19, with similar results (Fig. 1B). This result indicates that NMDA- and metabotropic glutamate

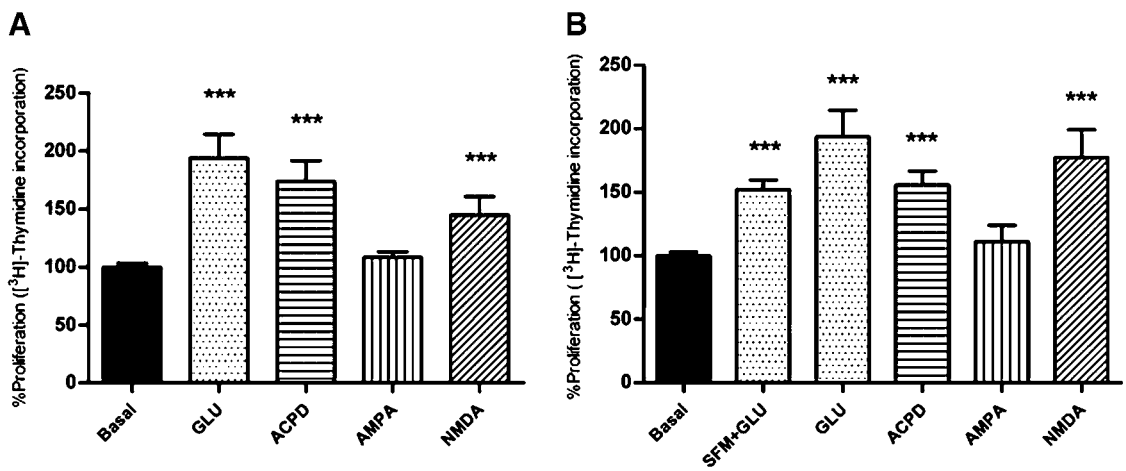

Fig. 1. Effect of glutamate and selective glutamate receptor agonists on RPE cell proliferation. Cells were cultured in the presence of 1.0 mM glutamate or 150 μM receptor agonists ACPD, AMPA, or NMDA for 24 h, followed by a further 16 h incubation with [^{3}H]-thymidine. **A**: Chick RPE; (**B**) ARPE-19 cell line. Results are expressed as the mean ± SEM of three independent experiments. ***$P < 0.001$, Student's *t*-test, compared to control. C, control; GLU, glutamate; SFM, serum free medium.

receptors are involved in glutamate-induced proliferation.

Glutamate-Induced Proliferation is Prevented by Specific Receptor Antagonists

The relative contribution of selective receptor subtypes to glutamate-induced proliferation was established by testing the effect of specific antagonists on glutamate-induced effect. Figure 2 shows that glutamate-induced proliferation was prevented by the mGluR antagonist MCPG and by the NMDAR open channel blocker MK-801. These results further demonstrate that glutamate stimulates proliferation by activating mainly NMDA- and Group I mGluRs. Unexpectedly, although AMPAR stimulation did not promote proliferation, the specific AMPA/KA receptor antagonist CNQX, prevented glutamate-induced proliferation. In addition to the inhibition of AMPARs, quinoxalines such as CNQX and NBQX have been shown to inhibit non-competitively NMDAR function due to an interaction with the glycine coagonist site of the NMDA receptor [Michaelis, 1998], which could explain this effect.

Glutamate Receptor Activation Stimulates ERK 1/2 Phosphorylation

The MAPK cascades and particularly the ERK1/2 signaling pathway are involved in the control of cell proliferation [Pearson et al., 2001]. We analyzed the effect of glutamate on ERK activation by means of an in vitro kinase-activity assay. Results show that stimulation by 1 mM glutamate transiently increases ERK1/2 phosphorylation in chick RPE and ARPE-19 cells: the response reached maximal value at 10 min, and slowly declined to basal level 30 min after stimulation (not shown). Consistent with the effect of glutamate agonists on proliferation (Fig. 1), results in Figure 3A show that ACPD and NMDA activate ERK 1/2, whereas AMPA has no effect. The mGluR antagonist MCPG and the NMDA open channel blocker MK-801 prevented ERK phosphorylation induced by glutamate (Fig. 3B). This result suggests that glutamate stimulates RPE proliferation through an increase in ERK phosphorylation, mediated by NMDA and Group-I metabotropic receptors. Interestingly, a single phospho-ERK protein band, corresponding to ERK2, was observed in the chick preparation. We hence tested the procedure in ARPE-19 cells in order to rule out a technical problem as the cause for this result. As shown in Figure 3C, the classical p44/p42 doublet was detected in these cells, suggesting a species-related difference in ERK expression.

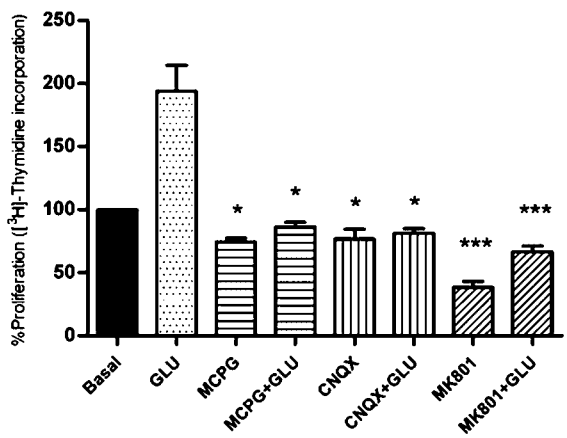

Fig. 2. Glutamate-induced RPE cell proliferation is inhibited by selective antagonists of glutamate receptor subtypes. Experiments were performed as described for Figure 1. Specific glutamate receptor subtype antagonists MCPG (50 μM), CNQX (10 μM), and MK-801 (10 μM) were included 3 h prior to 1.0 mM glutamate. [^{3}H]-hymidine was added 24 h after stimulation and maintained for 16 h. Results are expressed as the mean ± SEM of three independent experiments. ***$P < 0.001$, *$P < 0.05$ Student's *t*-test, compared to GLU.

ERK 1/2 Activation is Required for Glutamate-Induced RPE Cell Proliferation

In order to demonstrate a cause-effect relationship between glutamate-induced ERK phosphorylation and cell proliferation, we analyzed the effect of the MEK1/2 inhibitor PD98059 (30 μM) on proliferation and ERK activation, since ERK is the only known substrate for the upstream kinase MEK [Pearson et al., 2001]. As shown in Figure 4, whereas proliferation (panel A) and ERK phosphorylation (panel B) induced by mGluR stimulation were prevented by PD98059, the inhibition of MEK did not modify the effect of NMDA on either process (Fig. 4A,B), suggesting that glutamate activates two distinct signaling pathways leading to proliferation, linked to specific receptor subtypes: whereas mGluR stimulation induces proliferation by activating MEK/ERK, NMDAR stimulation promotes proliferation through a different mechanism, likely triggered by intracellular Ca^{++} increase.

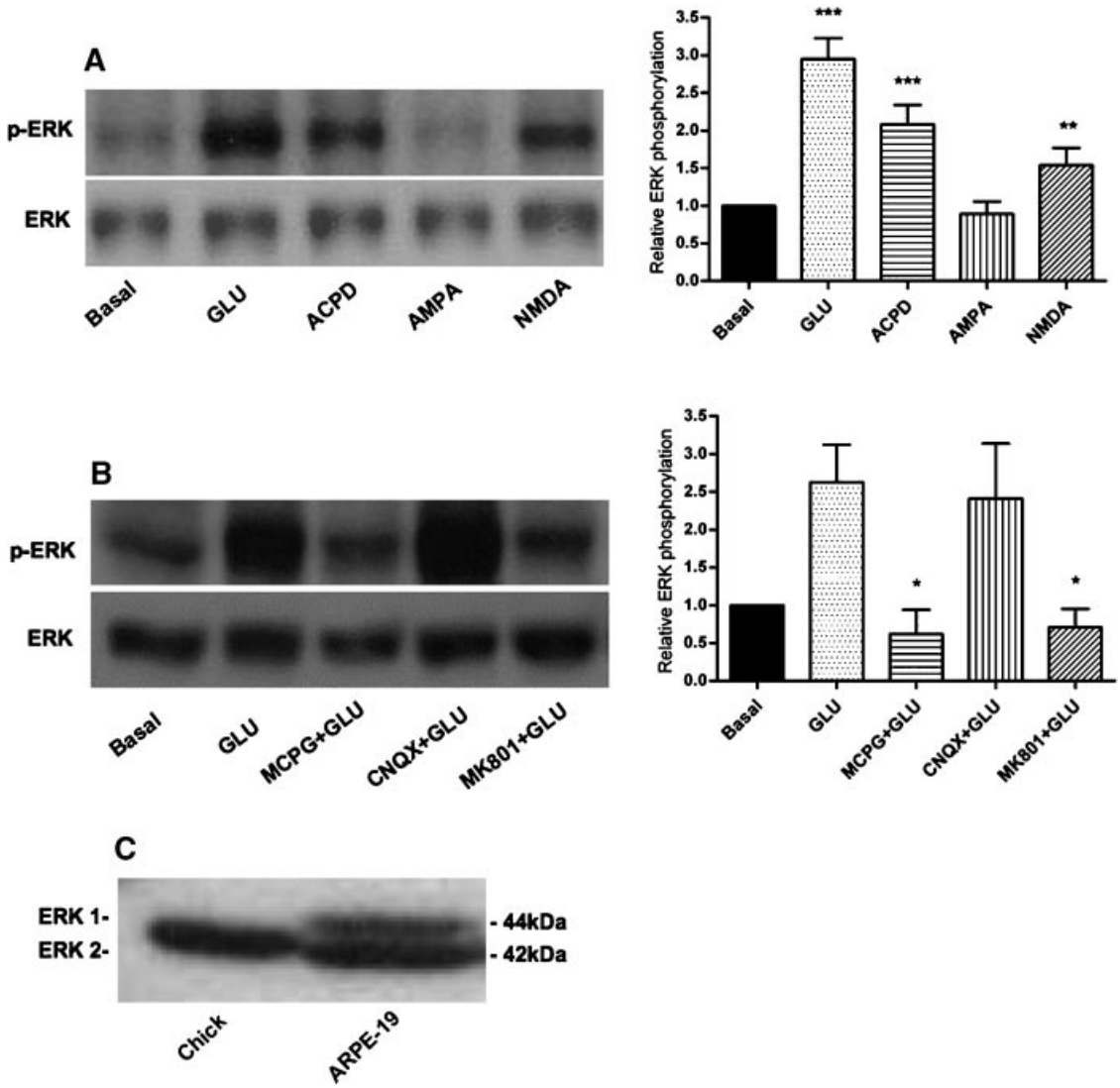

Fig. 3. Stimulation of selective glutamate receptor subtypes activates ERK1/2 in RPE cells. Experiments were performed as described in Methods, and for Figure 1. **A**: Specific glutamate agonists at mGluRs (ACPD) and iGluRs (NMDA and AMPA), differentially increase ERK1/2 phosphorylation. **B**: Stimulation by glutamate shown in (A) is prevented by the antagonists MCPG (mGluR) and MK-801 (NMDAR). The graphs represent the densitometric analysis of the immunoblots shown on the left. Values are expressed as the mean ± SEM of three independent experiments. **C**: Analysis of ERK 1/2 expression in chick and human-derived ARPE-19 RPE cells shows that ERK1 is not expressed in chick cells. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, Student's *t*-test, (A) referred to control; (B) referred to GLU.

CaMKs are Involved in Glutamate-Induced Proliferation

NMDA-induced ERK phosphorylation in neuronal cells has been shown to require CaMK activity [Illario et al., 2002; Schmitt et al., 2005]. Since NMDA-induced proliferation and ERK phosphorylation were not prevented by the inhibition of MEK (Fig. 4), we tested the effect of the CaMK inhibitors KN-62 and KN-93 on glutamate-, NMDA- and ACPD-induced proliferation and ERK phosphorylation. Results showed that 10 μM KN-62 not only prevents agonist-induced proliferation, but decreases proliferation beyond control values (Fig. 5A). As depicted in Figure 5B, ERK activation induced by ACPD was also inhibited by KN-93 suggesting that CaMKs contribute to mGluR/ERK signaling to proliferation. However, although the inhibition of CaMKs suppressed NMDA effect on proliferation (Fig. 5A), KN-93 did not prevent NMDA-induced ERK phosphorylation (Fig. 5B) which indicates that NMDA may stimulate proliferation through a distinct,

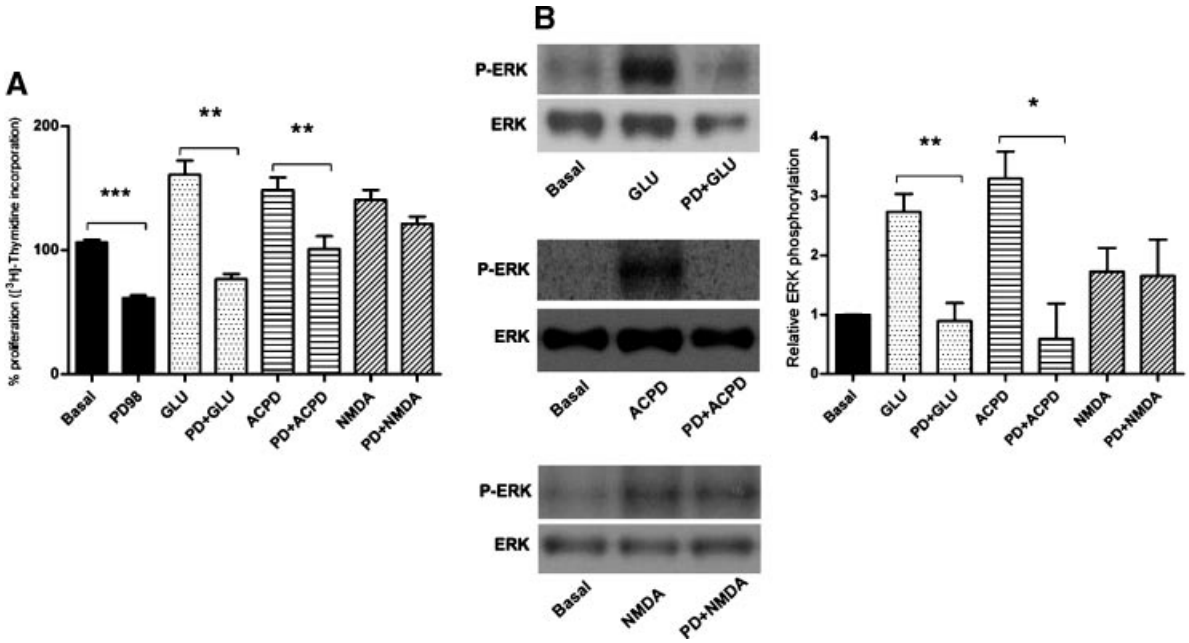

Fig. 4. Glutamate stimulates proliferation through ERK activation. Experiments were performed as described in Methods. **A**: Glutamate- and ACPD-induced cell proliferation is prevented by the MEK inhibitor PD98059 (PD, 30 μM). **B**: PD98059 prevents ERK phosphorylation induced by mGluR-activation but not by NMDAR-activation. The graph on B-right depicts the densitometric analysis of the ERK immunoblot on B-left. Results are expressed as the mean ± SEM of three independent experiments. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, significant difference from control in the absence of the inhibitor, Student's *t*-test.

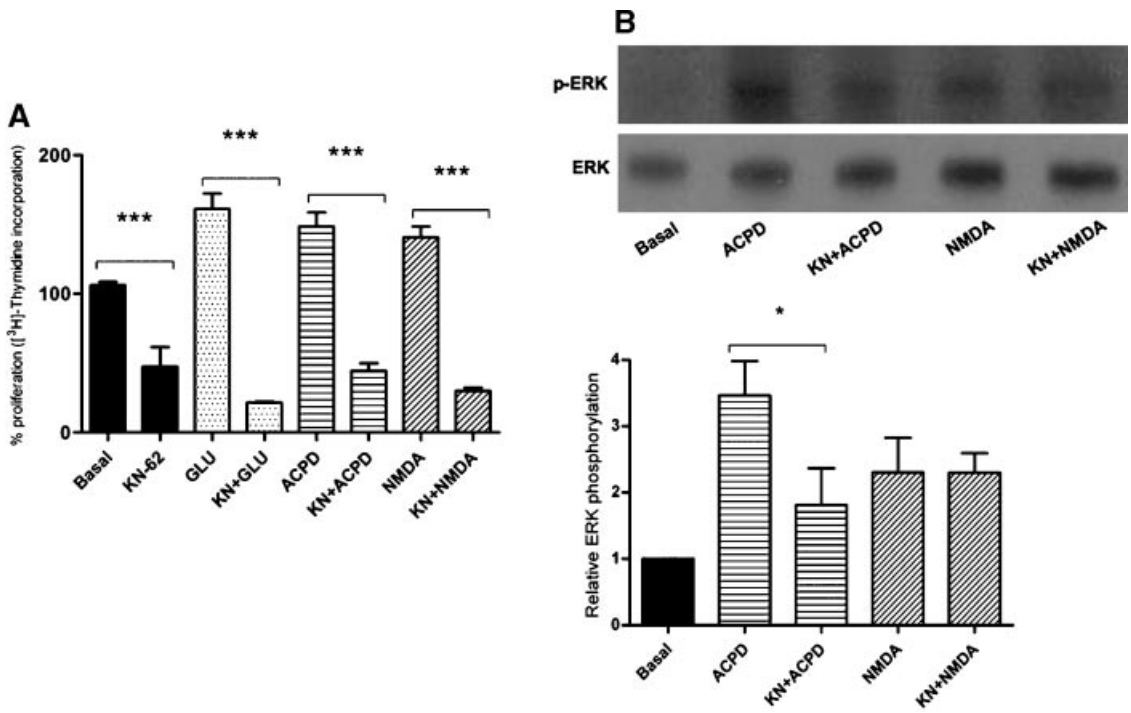

Fig. 5. CaMKs are involved in glutamate stimulation of RPE cell proliferation. Experiments were performed as described in Methods and for Figure 1. ERK activation was measured following 10 min stimulation by 150 μM ACPD or NMDA. KN-62 or KN-93 was included 3 h prior to agonist-stimulation. **A**: The CaMKs inhibitor KN-62 (KN, 10 μM) prevents the stimulation of proliferation induced by the activation of mGluRs (ACPD) and NMDARs. **B**: The CaMKs inhibitor KN-93 (10 μM) prevents the stimulation of ERK phosphorylation induced by ACPD, but not activation by NMDA. Results are expressed as the mean ± SEM of three independent experiments. $^{***}P < 0.001$, $^{*}P < 0.05$ Student's *t*-test, compared to control in the absence of the inhibitor.

MEK-independent signaling mechanism, mediated by CaMKs.

Glutamate Stimulates the Phosphorylation of CREB by ERK1/2

Glutamate stimulation of neuronal receptors has been shown to activate CREB by serine-133 phosphorylation carried by ERK 1/2 [Vanhoutte et al., 1999]; we hence analyzed the effect of glutamate on CREB phosphorylation. Results showed that CREB phosphorylation can be detected as early as 5 min following glutamate stimulation, reaches maximum value after 10 min, and decreases to control level at 30 min (Fig. 6A). This effect was mimicked by ACPD and NMDA, and blocked by the corresponding antagonists MCPG and MK-801, with equivalent potency to that observed for ERK activation (Fig. 6B).

Figure 7A shows that glutamate- and ACPD-induced CREB phosphorylation was prevented by the MEK1/2 inhibitor PD98059. Since NMDA-induced proliferation or ERK activation were not altered by the inhibition of MEK and, in contrast, NMDA-induced proliferation was prevented by CaMK inhibition, we tested the effect of KN-62 on NMDA-induced CREB phosphorylation; results in Figure 7B show the lack of effect of the CaMK inhibitor. These results further support that glutamate stimulates RPE cell proliferation by activating different pathways mediated by Group I metabotrpic receptors and NMDA receptors. Whereas the former involves the activation of MEK/ERK/CREB, the later requires the activity of CaMKs, and does not relate to the phosphorylation of ERK by MEK.

DISCUSSION

Numerous factors seem to be involved in the proliferation and transformation of RPE cells observed in PVR [Holtkamp et al., 2001; Hecquet et al., 2002]. Although glutamate

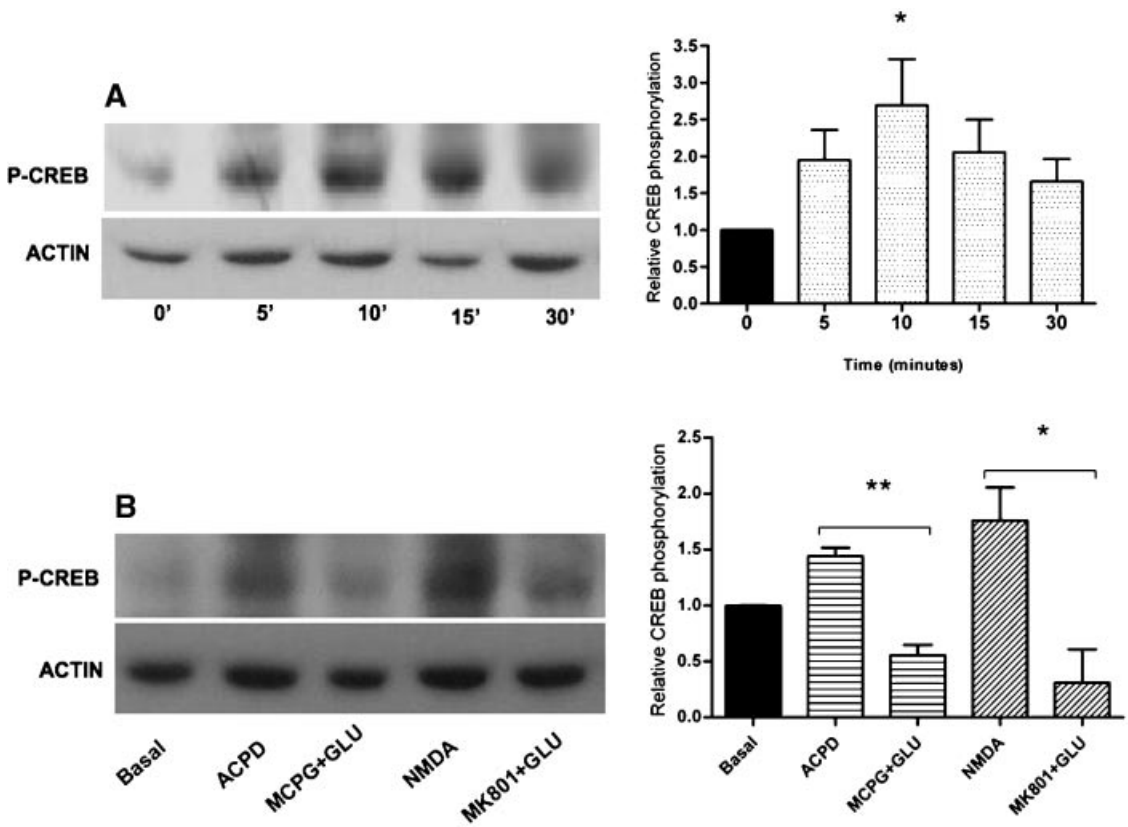

Fig. 6. Stimulation of metabotropic- and NMDA receptors induces the time-dependent activation of CREB. Experiments were performed as described in Methods. **A**: Glutamate (1 mM) stimulates CREB phosphorylation in a time-dependent manner. **B**: Glutamate-induced CREB activation is inhibited by the receptor inhibitors MCPG (mGluRs) and MK-801 (NMDARs). The graphs on the right depict the densitometric analysis of the blots on the left, normalized to actin. Results are expressed as the mean ± SEM of three independent experiments. $^{**}P < 0.01$, $^{*}P < 0.05$, Student's *t*-test.

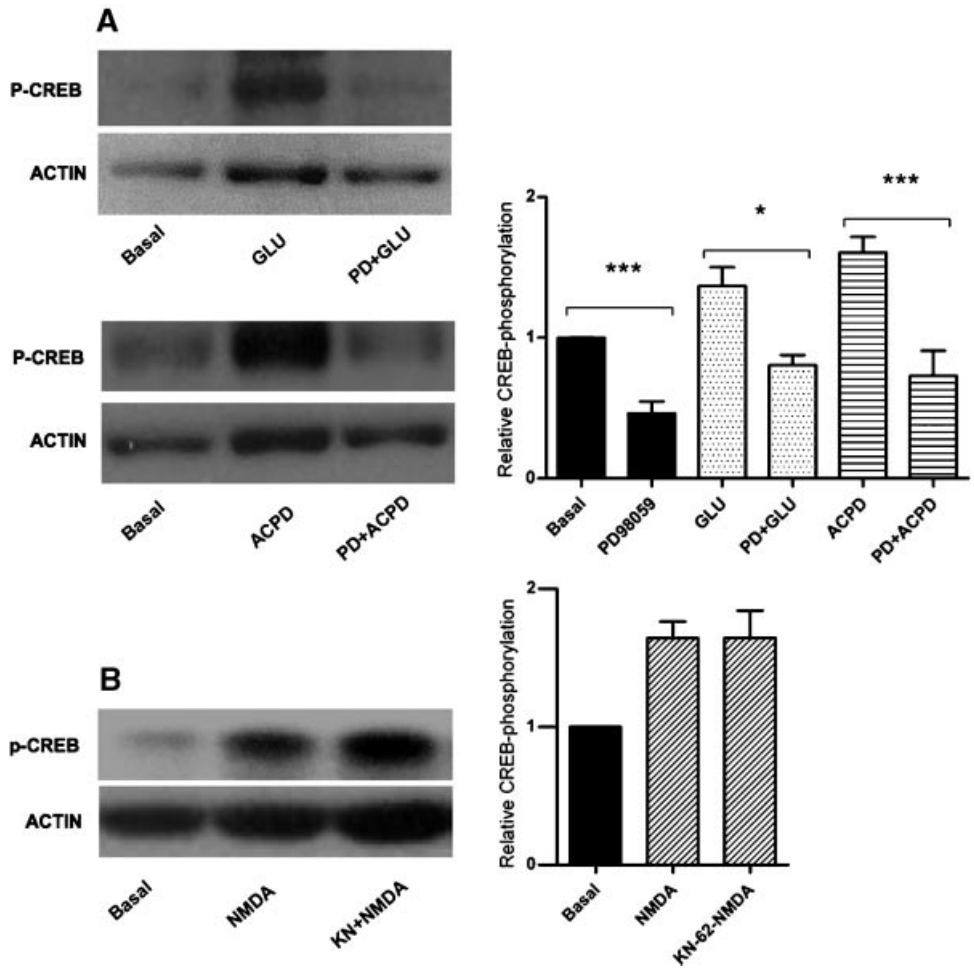

Fig. 7. Glutamate induces CREB phosphorylation by activating the MEK/ERK pathway. Experiments were performed as described in Methods. **A**: The MEK inhibitor PD98059 (30 μM) was included in the medium 3 h prior to the addition of the agonists. The graph on the right corresponds to the densitometric analysis of the immunoblots shown on the left, normalized to actin. **B**: KN-62 fails to inhibit the NMDA-induced CREB phosphorylation. Results are the mean ± SEM of three independent experiments. $^{***}P < 0.001$; $^{*}P < 0.05$, Student's *t*-test.

concentration in PVR tissue has not been determined, an increase in glutamate levels in the vitreous cavity of patients has been observed following retinal detachment and hypoxia [Diederen et al., 2006]. Such increase could be due to the decrease in the expression of the transporter proteins responsible for its clearance [Lewis et al., 1989; Li and Puro, 2002; Gadea et al., 2004] in retinal glia, a core component of PVR epiretinal membranes, which have also been shown to release glutamate in response to receptor-induced osmotic stress [Takano et al., 2005]. Additionally, upon retinal detachment or surgical damage to the BRB, glutamate from serum (100–300 μM), or from damaged retinal cells which contain millimolar glutamate concentrations, may reach the RPE [Castillo et al., 1997]. Hence, under pathologic conditions in vivo, elevation of glutamate could contribute to the development of PVR through the activation of specific receptors on RPE cells.

The stimulation of ionotropic and metabotropic glutamate receptors in neurons has been shown to activate the PKC-, PI3-K-, and Ca^{++}/calmodulin-dependent Ras/Raf/MEK/ERK cascade [Wang et al., 2007], known to participate in the regulation of proliferation, differentiation and survival processes [López-Colomé and Ortega, 1997; Pearson et al., 2001; Thomas and Huganir, 2004; Wang et al., 2004]. In RPE, although a previous report showed an indirect effect of glutamate on RPE proliferation [Uchida et al., 1998], and results from Hecquet et al. [2002] have shown the involvement of the Ras/Raf/MEK/ERK MAPK pathway in

serum-induced RPE proliferation, neither the activation of this pathway by glutamate, nor the increase in proliferation induced by such activity has been previously established in RPE.

We demonstrated here that glutamate increases the proliferation of chick RPE and human-derived ARPE-19 cells by activating two signaling pathways linked to NMDARs and Group I mGluRs, since this effect was prevented by the receptor-specific inhibitors MK-801 and MCPG, respectively. Whereas the effect of mGluR stimulation by glutamate and ACPD on proliferation was prevented by the MEK inhibitor PD98059, indicating the participation of the MEK/ERK1/2 pathway (Fig. 4), NMDAR-induced proliferation was prevented by the CaMK inhibitor KN-62, but not by PD98059, suggesting the activation by CaMKs of an alternate pathway leading to proliferation, independent from MEK/ERK.

In order to support this conclusion, we measured the effect of NMDAR- and mGluR stimulation on the activation of ERK1/2. Although ACPD and NMDA were shown to increase the level of phosphorylated ERK to similar extent, ACPD-induced effect was prevented by PD98059, suggesting ERK activation by MEK, whereas the NMDA-induced increase in phosphorylated ERK was not modified by PD-98059 or by KN-93.

The lack of effect of PD98059 and KN-93 on NMDA-induced ERK phosphorylation rules out the activation of the MEK/ERK pathway [Vanhoutte et al., 1999] and suggests that the elevation of phosphorylated ERK might, alternatively, derive from decreased dephosphorylation due to the inactivation of MAPK phosphatases by the inhibition of a CaMK-mediated activating mechanism [Bito et al., 1996; Lin and Yang, 2006]. Together with the inhibition of NMDA-induced activation of ERK by MK-801, the failure of PD98059 to inhibit NMDA effect rules-out the possible activation of Tyrosine Kinase receptors by neurotrophins such as BDNF, PDGF, or FGF [Uchida et al., 1998], which could be released by Ca^{2+} entry through NMDARs, as a possible cause of this effect.

We showed that, in addition to PD98059, the activation of ERK by mGluRs was also decreased by KN93, which may be due to the activation of PLC, the formation of IP3 [Fragoso and López-Colomé, 1999], the increase in cytoplasmic calcium, and the subsequent activation of CaMKs. Together these results demonstrate that although the induction of cell proliferation by mGluR activation is mainly achieved through the MEK/ERK pathway, the activation of CaMKs contributes to this effect.

On this matter, a role for CaMKs in the regulation of the Ras/Raf/MEK/ERK pathway has been documented, although the precise CaMKs involved are not clearly defined. CaMKII has been suggested to play an important role in the activation of ERK by NMDA receptors in striatal neurons, through the activation of Ras [Vanhoutte et al., 1999; Choe and Wang, 2001], and also to modulate proliferation mediated by this pathway by activating the upstream kinase Raf-1 [Illario et al., 2002]. In addition, the possible contribution of CaMKK and CaMKI and of PI3K to ERK phosphorylation in our system cannot be excluded, since the activation of CaMKI by CaMKK in neuronal cells has been shown to mediate depolarization- and NMDA-induced ERK activation [Soderling and Stull, 2001; Uezu et al., 2002; Schmitt et al., 2004]. Furthermore, the phosphorylation of ERK by NMDAR activation during E-LTP was shown to require CaMKK acting through CaMKI [Schmitt et al., 2005]. Likewise, recent reports have also suggested the activation of ERK1/2 signaling pathway by a PI3 kinase-dependent mechanism [Agell et al., 2002; Perkinton et al., 2002].

In order to determine the downstream target of activated ERK, the effect of glutamate and ACPD on CREB phosphorylation was first analyzed. In agreement with previous work showing that, among multiple factors, MAPK cascade activates CREB [Xing et al., 1996], a fast and transient activation of CREB in correlation with the time-course of ERK activation was observed, which was inhibited by PD98059 (Fig. 7A), indicating that mGluR-induced CREB phosphorylation results from MEK-induced ERK activation.

Since the effect of NMDAR-stimulation on proliferation and ERK phosphorylation was not modified by PD98059, we next tested the effect of KN-62, which inhibited NMDA-induced proliferation, on NMDA-induced CREB activation and found that NMDA-induced CREB activation was not modified by this agent. Although further investigation is required to support this notion, a possible explanation for this result could be the inhibition of calcineurin, a Ca^{++}/CaM-dependent Ser/Thr phosphatase shown

to dephosphorylate CREB at Ser 133, by the KN-62 inhibition of CaMKs [Shaywitz and Greenberg, 1999].

Intracellular calcium increase activates numerous, often opposing signaling pathways. These multiple actions depend, among other factors, on the route of calcium entry [Shaywitz and Greenberg, 1999]. In neurons, calcium influx through synaptic NMDARs activates two key signaling pathways, both of which lead to CREB phosphorylation on Ser133: the Ras-ERK1/2 pathway and the nuclear CaMK pathway, probably involving CaM kinase IV [Agell et al., 2002]. CaMK IV is calcium-dependent, whereas ERK1/2 and RSK2 are not, and consequently are activated more slowly than CaMKs and their activity is sustained. Thus, although the CaMK pathway activates CREB within the first few seconds of Ca^{++} influx, the recruitment of ERK1/2 pathway is needed to prolong CREB phosphorylation after activity has ceased, which is important for robust activation of CREB-dependent gene expression, which possibly requires the activation of CBP by CaMK IV phosphorylation of Ser301 [Deisseroth et al., 1998; Hardingham and Bading, 2003].

In our model, the increase in phosphorylated CREB either by MEK/ERK activation (mGluR), or by decreased ERK dephosphorylation (NMDAR), could promote the expression of genes involved in cell cycle progression [Chang et al., 2003; Roux and Blenis, 2004], thus stimulating proliferation. Furthermore, an increase in cyclin D1 expression due to ERK activation has been recently shown to increase the proliferation of RPE cells induced by serum [Hecquet et al., 2002]. The fact that KN-62 did not inhibit CREB phosphorylation induced by either receptor further indicates that CaMKs participate in glutamate receptor-induced cell proliferation at a step downstream of CREB, consistent with an action on cell cycle regulation.

We found that stimulation of proliferation by ACPD (mGluR) was inhibited by PD98059, whereas that induced by NMDA was not, however, the effect of both agonists on proliferation was completely blocked by KN-62, suggesting that, in addition to the possible activation of Ras by CaMKs due to calcium increase driven by mGluRs, CaMKs also participate in the regulation of proliferation induced by both receptor subtypes at a level downstream of CREB, possibly the regulation of cell cycle.

The Ca^{++}/CaM-dependent pathways which regulate progression through cell cycle transitions remain ambiguous however, the transcriptional activation of specific genes involved in this process requires CaMKs activity [Kahl and Means, 2003]. The induction of proliferation requires, particularly, the expression of cyclins which control cell cycle progression by interacting with- and activating cyclin-dependent kinases (CDKs). Our results showing that KN-62 not only inhibits mGluR-induced proliferation, but further decreases the basal (control) proliferation rate of RPE cells (Fig. 5A), are in agreement with previous reports showing that KN-93 prevents G1 cell cycle phase progression in both, normal and

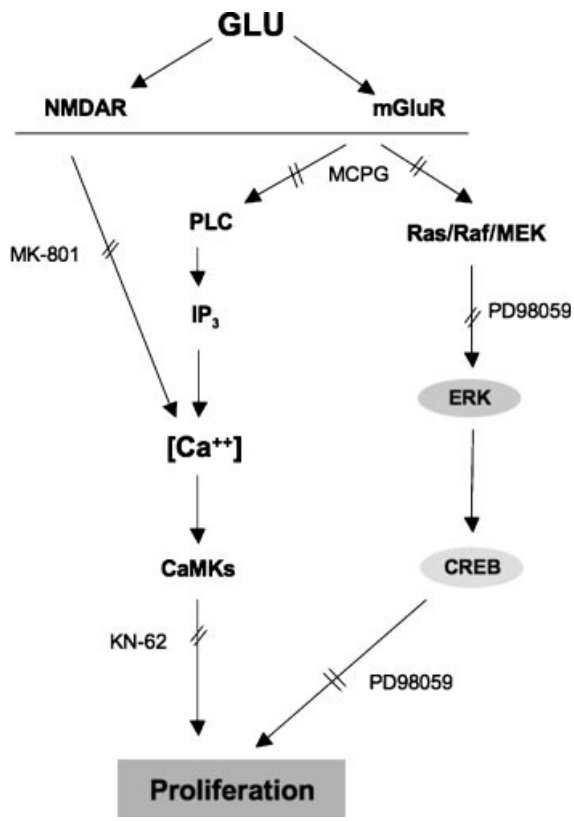

Fig. 8. Schematic of GluR-mediated stimulation of RPE cell proliferation. The activation of mGluRs and ionotropic NMDARs by increased extracellular glutamate stimulates cell proliferation by activating distinct signaling pathways. The inhibition of MEK prevents mGluR- but not NMDAR-mediated stimulation of proliferation. Inhibition of CaMKs prevents the increase in proliferation induced by both, mGluR and NMDAR stimulation, suggesting the concurrence of these pathways downstream of CREB, possibly at the regulation of cell cycle progression (see Discussion).

transformed cells [Kahl and Means, 2003; Choi and Husain, 2006]. This possibility would also explain the inhibition of NMDA-induced proliferation by KN-62, due to the NMDA-mediated calcium increase and the consequent activation of CaMKs. Although KN-62 is suggested to be CaMKII-specific, KN-62 actually inhibits CaMKI and CaMKII with similar efficacy [Hidaka and Yokokura, 1996], and CaMKI, rather than CaMKII has been proposed to regulate the progression of G1 phase of the cell cycle [Kahl and Means, 2003].

The present findings demonstrate that glutamate activates two parallel pathways leading to ERK1/2 and CREB phosphorylation in RPE cells by acting on metabotropic (Group I) and NMDA receptor subtypes, and also show that the independent stimulation of both pathways increases RPE cell proliferation (Fig. 8). These results thus suggest that under pathological conditions or surgical procedures in which the BRB is compromised, the elevation of extracellular glutamate may be involved in the alteration of gene expression leading to the long term increase in RPE cell proliferation, as observed in PVR.

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REFERENCES

Abe T, Durlu YK, Tamai M. 1996. The properties of retinal pigment epithelial cells in proliferative vitreoretinopathy compared with cultured retinal pigment epithelial cells. Exp Eye Res 63:201–210.

Agell N, Bacha O, Rocamora N, Villalonga P. 2002. Modulation of the Ras/Raf/MEK/ERK pathway by Ca^{2+}, and calmodulin. Cell Signal 14:649–654.

Ando A, Ueda M, Uyama M, Masu Y, Ito S. 2000. Enhancement of dedifferentiation and myoid differentiation of retinal pigment epithelial cells by platelet derived growth factor. Br J Ophthalmol 84:1306–1311.

Bito H, Deisseroth K, Tsien RW. 1996. CREB Phosphorylation and dephosphorylation: A Ca^{2+}- and stimulus duration-dependent switch for hippocampal gene expression. Cell 87:1203–1214.

Bryckaert M, Guillonneau X, Hecquet C, Perani P, Courtois Y, Mascarelli F. 2000. Regulation of proliferation-survival decisions is controlled by FGF1 secretion in retinal pigmented epithelial cells. Oncogene 19:4917–4929.

Casaroli-Marano RP, Pagan R, Vilaro S. 1999. Epithelial-mesenchymal transition in proliferative vitreoretinopathy: Intermediate filament protein expression in retinal pigment epithelial cells. Invest Ophthalmol Vis Sci 40: 2062–2072.

Castillo J, Davalos A, Noya M. 1997. Progression of ischaemic stroke and excitotoxic aminoacids. Lancet 349:79–83.

Chambard JC, Lefloch R, Pouyssegur J, Lenormand P. 2007. ERK implication in cell cycle regulation. Biochim Biophys Acta 1773:1299–1310.

Chang F, Steelman LS, Shelton JG, Lee JT, Navolanic PM, Blalock WL, Franklin R, McCubrey JA. 2003. Regulation of cell cycle progression and apoptosis by the Ras/Raf/MEK/ERK pathway (Review). Int J Oncol 22:469–480.

Choe ES, Wang JQ. 2001. Group I metabotropic glutamate receptors control phosphorylation of CREB, Elk-1 and ERK via a CaMKII-dependent pathway in rat striatum. Neurosci Lett 313:129–132.

Choi J, Husain M. 2006. Calmodulin-mediated cell cycle regulation. Cell Cycle 5:2183–2186.

Deisseroth K, Heist EK, Tsien RW. 1998. Calmodulin translocation to the nucleus supports CREB phosphorylation in hippocampal neurons. Nature 392:198–202.

Diederen RM, La Heij EC, Deutz NE, Kijlstra A, Kessels AG, van Eijk HM, Liem AT, Dieudonne S, Hendrikse F. 2006. Increased glutamate levels in the vitreous of patients with retinal detachment. Exp Eye Res 83:45–50.

Dunn KC, Aotaki-Keen AE, Putkey FR, Hjelmeland LM. 1996. ARPE-19, a human retinal pigment epithelial cell line with differentiated properties. Exp Eye Res 62:155–169.

Fragoso G, López-Colomé AM. 1999. Excitatory amino acid-induced inositol phosphate formation in cultured retinal pigment epithelium. Vis Neurosci 16:263–269.

Frödin M, Gammeltoft S. 1999. Role and regulation of 90 kDa ribosomal S6 kinase (RSK) in signal transduction. Mol Cell Endocrinol 151:65–77.

Fukunaga K, Miyamoto E. 1998. Role of MAP kinase in neurons. Mol Neurobiol 16:79–95.

Gadea A, Lopez E, López-Colomé AM. 2004. Glutamate-induced inhibition of D-aspartate uptake in Muller glia from the retina. Neurochem Res 29:295–304.

Grisanti S, Guidry C. 1995. Transdifferentiation of retinal pigment epithelial cells from epithelial to mesenchymal phenotype. Invest Ophthalmol Vis Sci 36:391–405.

Guerrini L, Blasi F, Denis-Donini S. 1995. Synaptic activation of NF-kappa B by glutamate in cerebellar granule neurons in vitro. Proc Natl Acad Sci USA 92:9077–9081.

Haddad JJ. 2005. N-methyl-D-aspartate (NMDA) and the regulation of mitogen-activated protein kinase (MAPK) signaling pathways: A revolving neurochemical axis for therapeutic intervention? Prog Neurobiol 77:252–282.

Hardingham GE, Bading H. 2003. The Yin and Yang of NMDA receptor signaling. Trends Neurosci 26:81–89.

Hecquet C, Lefevre G, Valtink M, Engelmann K, Mascarelli F. 2002. Activation and role of MAP kinase-dependent pathways in retinal pigment epithelial cells: ERK and RPE cell proliferation. Invest Ophthalmol Vis Sci 43: 3091–3098.

Hidaka H, Yokokura H. 1996. Molecular and cellular pharmacology of a calcium/calmodulin-dependent protein

kinase II (CaM kinase II) inhibitor, KN-62, and proposal of CaM kinase phosphorylation cascades. Adv Pharmacol 36:193–219.

Holtkamp GM, Kijlstra A, Peek R, de Vos AF. 2001. Retinal pigment epithelium-immune system interactions: Cytokine production and cytokine-induced changes. Prog Retin Eye Res 20:29–48.

Illario M, Cavallo AL, Bayer KU, Di Matola T, Fenzi G, Rossi G, Vitale M. 2002. Calcium/calmodulin-dependent protein kinase II binds to Raf-1 and modulates integrin-stimulated ERK activation. J Biol Chem 278:45101–45108.

Isenoumi K, Kumai T, Kitaoka Y, Motoki M, Kitaoka Y, Kuribayashi K, Munemas Y, Kogo J, Kobayashi S, Ueno S. 2004. N-Methyl-D-aspartate induces phosphorylation of cAMP response element (CRE)-binding protein and increases DNA-binding activity of CRE in rat retina. J Pharmacol Sci 95:108–114.

Kahl CR, Means A. 2003. Regulation of cell cycle progression by calcium/calmodulin-dependent pathways. Endocr Rev 24:719–736.

Kaven CW, Spraul CW, Zavazava NK, Lang GK, Lang GE. 2000. Growth factor combinations modulate human retinal pigment epithelial cell proliferation. Curr Eye Res 20:480–487.

Komuro H, Rakic P. 1993. Modulation of neuronal migration by NMDA receptors. Science 260:95–97.

Lewis GP, Erickson PA, Guerin CJ, Anderson DH, Fisher SK. 1989. Changes in the expression of specific Muller cell proteins during long-term retinal detachment. Exp Eye Res 49:93–111.

Li Q, Puro DG. 2002. Diabetes-induced dysfunction of the glutamate transporter in retinal Muller cells. Invest Ophthalmol Vis Sci 43:3109–3116.

Limb GA, Little BC, Meager A, Ogilvie JA, Wolstencroft RA, Franks WA, Chignell AH, Dumonde DC. 1991. Cytokines in proliferative vitreoretinopathy. Eye 5(Pt 6):686–693.

Lin Y-W, Yang J-L. 2006. Cooperation of ERK and SCF^{Skp2} for MKP-1 destruction provides a positive feedback regulation of proliferation signaling. J Biol Chem 281:915–926.

López-Colomé AM, Ortega A. 1997. Activation of p42 mitogen-activated protein kinase by glutamate in cultured radial glia. Neurochem Res 22:679–685.

López-Colomé AM, Salceda R, Fragoso G. 1993. Specific interaction of glutamate with membranes from cultured retinal pigment epithelium. J Neurosci Res 34:454–461.

López-Colomé AM, Fragoso G, Wright CE, Sturman JA. 1994. Excitatory amino acid receptors in membranes from cultured human retinal pigment epithelium. Curr Eye Res 13:553–560.

Mao L, Tang Q, Samdani S, Liu Z, Wang JQ. 2004. Regulation of MAPK/ERK phosphorylation via ionotropic glutamate receptors in cultured rat striatal neurons. Eur J Neurosci 19:1207–1216.

Massey SC, Miller RF. 1990. N-methyl-D-aspartate receptors of ganglion cells in rabbit retina. J Neurophysiol 63:16–30.

Michaelis EK. 1998. Molecular biology of glutamate receptors in the central nervous system and their role in excitotoxicity, oxidative stress and aging. Prog Neurobiol 54:369–415.

Musgrove EA. 2006. Cyclins: Roles in mitogenic signaling and oncogenic transformation. Growth Factors 24:13–19.

Pastor JC, de la Rua ER, Martin F. 2002. Proliferative vitreoretinopathy: Risk factors and pathobiology. Prog Retin Eye Res 21:127–144.

Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K, Cobb MH. 2001. Mitogen-activated protein (MAP) kinase pathways: Regulation and physiological functions. Endocr Rev 22:153–183.

Perkinton MS, Ip JK, Wood GL, Crossthwaite AJ, Williams RJ. 2002. Phosphatidylinositol 3-kinase is a central mediator of NMDA receptor signalling to MAP kinase (Erk1/2), Akt/PKB and CREB in striatal neurones. J Neurochem 80:239–254.

Roux PP, Blenis J. 2004. ERK and p38 MAPK-activated protein kinases: A family of protein kinases with diverse biological functions. Microbiol Mol Biol Rev 68:320–344.

Schmitt JM, Wayman GA, Nozaki N, Soderling TR. 2004. Calcium activation of ERK mediated by calmodulin kinase I. J Biol Chem 279:24064–24072.

Schmitt JM, Gire ES, Saneyoshi T, Soderling TR. 2005. Calmodulin-dependent kinase kinase/calmodulin kinase I activity gates extracellular-regulated kinase-dependent long-term potentiation. J Neurosc 25:1281–1290.

Shaulian E, Karin M. 2002. AP-1 as a regulator of cell life and death. Nat Cell Biol 4:E131–E136.

Shaywitz AJ, Greenberg ME. 1999. CREB: A stimulus-induced transcription factor activated by a diverse array of extracellular signals. Annu Rev Biochem 68:821–861.

Siu YT, Jin DY. 2007. CREB-areal culprit in oncogenesis. FEBS J 274:3224–3232.

Soderling TR, Stull JT. 2001. Structure and regulation of calcium/calmodulin-dependent protein kinases. Chem Rev 101:2341–2352.

Stepulak A, Sifringer M, Rzeski W, Endesfelder S, Gratopp A, Pohl EE, Bittigau P, Felderhoff-Mueser U, Kaindl AM, Buhrer C, Hansen HH, Stryjecka-Zimmer M, Turski L, Ikonomidou C. 2005. NMDA antagonist inhibits the extracellular signal-regulated kinase pathway and suppresses cancer growth. Proc Natl Acad Sci USA 102: 15605–15610.

Strauss O. 2005. The retinal pigment epithelium in visual function. Physiol Rev 85:845–881.

Takano T, Kang J, Jaiswal JK, Simon SM, Lin JH, Yu Y, Li Y, Yang J, Dienel G, Zielke HR, Nedergaard M. 2005. Receptor-mediated glutamate release from volume sensitive channels in astrocytes. Proc Natl Acad Sci USA 102:16466–16471.

Thomas GM, Huganir RL. 2004. MAPK cascade signalling and synaptic plasticity. Nat Rev Neurosci 5:173–183.

Uchida N, Kiuchi Y, Miyamoto K, Uchida J, Tobe T, Tomita M, Shioda S, Nakai Y, Koide R, Oguchi K. 1998. Glutamate-stimulated proliferation of rat retinal pigment epithelial cells. Eur J Pharmacol 343:265–273.

Uezu A, Fukunaga K, Kasahara J, Miyamoto E. 2002. Activation of Ca^{2+}/calmodulin-dependent protein kinase I in cultured rat hippocampal neurons. J Neurochem 82:585–593.

Vanhoutte P, Barnier JV, Guibert B, Pages C, Besson MJ, Hipskind RA, Caboche J. 1999. Glutamate induces phosphorylation of Elk-1 and CREB, along with c-fos activation, via an extracellular signal-regulated kinase-dependent pathway in brain slices. Mol Cell Biol 19:136–146.

Wang JQ, Tang Q, Parelkar NK, Liu Z, Samdani S, Choe ES, Yang L, Mao L. 2004. Glutamate signaling to Ras-MAPK in striatal neurons: Mechanisms for inducible gene expression and plasticity. Mol Neurobiol 29:1–14.

Wang JQ, Fibuch EE, Mao L. 2007. Regulation of mitogen-activated protein kinases by glutamate receptors. J Neurochem 100:1–11.

Xing J, Ginty DD, Greenberg ME. 1996. Coupling of the ras-MAPK pathway to gene activation by RSK2, a growth-factor-regulated CREB kinase. Science 273: 959–963.

Xu L, Deng X. 2006. Suppression of cancer cell migration and invasion by protein phosphatase 2A through dephosphorylation of mu- and m-calpains. J Biol Chem 281: 35567–35575.

Yoon S, Seger R. 2006. The extracellular signal-regulated kinase: Multiple substrates regulate diverse cellular functions. Growth Factors 24:21–44.