Differential regulation of metabotropic glutamate receptor 5-mediated phosphoinositide hydrolysis and extracellular signal-regulated kinase responses by protein kinase C in cultured astrocytes

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Abstract
The metabotropic glutamate receptor 5 (mGluR5) exhibits a rapid loss of receptor responsiveness to prolonged or repeated agonist exposure. This receptor desensitization has been seen in a variety of native and recombinant systems, and is thought to result from receptor-mediated, protein kinase C (PKC)-dependent phosphorylation of the receptor, uncoupling it from the G protein in a negative feedback regulation. We have investigated the rapid PKC-mediated desensitization of mGluR5 in cortical cultured astrocytes by measuring downstream signals from activation of mGluR5. These include activation of phosphoinositide (PI) hydrolysis, intracellular calcium transients, and extracellular signal-regulated kinase 2 (ERK2) phosphorylation. We present evidence that PKC plays an important role in rapid desensitization of PI hydrolysis and calcium signaling, but not in ERK2 phosphorylation. This differential regulation of mGluR5-mediated responses suggests divergent signaling and regulatory pathways which may be important mechanisms for dynamic integration of signal cascades.

Keywords: astrocytes, desensitization, extracellular signal-regulated kinase 2, metabotropic glutamate receptor 5, phosphoinositide hydrolysis, protein kinase C. J. Neurochem. (2002) 83, 110–118.

Desensitization, the phenomenon of diminished receptor responsiveness in the continued presence of agonist is a common characteristic of G protein-coupled receptors (GPCR). Despite the commonalities, the molecular mechanisms of desensitization often appear as a complex interplay of receptors, effectors, second messengers, accessory and scaffolding proteins, and protein kinases. These multiple mechanisms of receptor desensitization can lend specificity to regulation of GPCR signaling. Receptor phosphorylation, mediated by second messenger kinases such as protein kinase C (PKC) and cAMP-dependent protein kinase (PKA), or G protein-coupled receptor kinases (GRK) plays a key role in desensitization as an immediate effect of receptor activation and results in rapid reduction in signaling responsiveness (Chuang et al. 1996; Freedman and Leifkowitz 1996). The physiological consequences of desensitization include the rapid feedback regulation of G protein coupling, as well as longer term effects of receptor internalization and down-regulation and activation of other signaling pathways (Ferguson 2001).
Metabotropic glutamate receptors (mGluRs) have been classified into three major groups based on sequence homology, pharmacology, and second messenger coupling (Conn and Pin 1997). In expression systems and native tissue preparations, group-I mGluRs (mGluR1 and mGluR5) couple to Gαq/11 and activation of phospholipase Cβ (PLCβ) and phosphoinositide (PI) hydrolysis, yielding diacylglycerol (DAG), which activates protein kinase C (PKC), and inositol trisphosphate (IP3) which elevates intracellular calcium by triggering its release from internal stores. Group-II and III mGluRs couple to Gi/o and the inhibition of adenylyl cyclase. In addition, mGluRs have been shown to activate a variety of other effector systems through direct and indirect mechanisms. Examples include activation of phospholipase D, potentiation of Gs-linked increases in cyclic AMP, activation of phospholipase A2 and release of arachidonic acid, and increases in cyclic GMP (reviewed in Conn and Pin 1997). More recently, stimulation of mGluRs has been shown to lead to phosphorylation of mitogen-activated protein (MAP) kinases (Peavy and Conn 1998; Ferraguti et al. 1999), and a G protein-independent activation of a Src-family protein tyrosine kinase as a component of a slow excitatory postynaptic current (Heuss et al. 1999).

Group-I mGluRs also exhibit rapid receptor desensitization (for reviews see Alagarsamy et al. 2001; De Blasi et al. 2000). Prolonged or repeated exposures to agonists decreased mGluR-mediated PI hydrolysis in neuronal (Nicoletti et al. 1986; Catania et al. 1991) and astrocytic (Baláz et al. 1997) cultures, and also decreased diacylglycerol (DAG) production in synaptosomes (Herrero et al. 1994, 1998). In addition, evidence from studies for several years suggested that PKC plays a role in a feedback regulation of group-I mGluR responsiveness. In several native preparations, prior activation of PKC with phorbol esters caused an inhibition of mGluR-mediated PI hydrolysis. Incubation with PKC inhibitors reversed this inhibition, often potentiating the response (Canonico et al. 1988; Schoepf and Johnson 1988; Godfrey and Taghavi 1990; Manzoni et al. 1990). These effects were also observed in transiently and stably transfected cell lines expressing mGluR1a (Thomsen et al. 1993; Francescon and Duvoisin 2000) and mGluR1c (Ciruela et al. 1999), often accompanied by PKC-mediated receptor phosphorylation (Alalu et al. 1995; Ciruela et al. 1999). Studies using Xenopus oocytes expressing wild-type and PKC consensus site mutants of mGluR5 also identified a rapid, PKC-mediated desensitization of PLC-dependent, calcium activated chloride currents (Gereau and Heinemann 1998; Alagarsamy et al. 1999). The mGluR5 desensitization was also evident in neurons and was accompanied by agonist-mediated phosphorylation of the receptor (Alagarsamy et al. 1999). Finally, in our recent report, we described similar rapid and transient kinetics for mGluR5-mediated ERK2 phosphorylation in cortical astrocytes, which reaches a maximum within 5 min, and declines to basal levels 1 h after agonist application (Peavy and Conn 1998). Yet in many of these reports, PKC-mediated effects could not account for all aspects of mGluR desensitization (Catania et al. 1991; Baláz et al. 1997; Alagarsamy et al. 1999). In addition, recent reports have also identified GRK-mediated mechanisms of mGluR desensitization (Dale et al. 2000; Sallese et al. 2000).

The focus of this study was to examine the role of PKC-mediated receptor phosphorylation in the rapid desensitization of mGluR5-mediated signals in cortical astrocytes. We measured PI hydrolysis, calcium transients and ERK2 phosphorylation during the initial minutes following agonist application. Our studies suggest that PLCβ-dependent responses are regulated by PKC, at least in part, while ERK2 phosphorylation is independent of PKC regulation.

Materials and methods

Materials

The following chemicals and reagents were obtained from the indicated sources: (R,S)-3,5-dihydroxystyrylglycine (DHGP), pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid tetrasodium salt (PPADS), and 6-cyano-7-nitroquinoxaline-2,3-dione disodium salt (CNQX) from Tocris-Cookson Inc., Ballwin, MO, USA; bisindolylmaleimide I (BIS I) hydrochloride, Ro-31–8220, and BIS V, from Calbiochem, San Diego, CA; phorbol 12,13-dibutyrate (PDBu) and 4z-phorbol from Sigma, St Louis, MO, USA; and Fluo-3 AM fluorescent calcium indicator from Molecular Probes, Eugene, OR, USA. [3H]Myo-inositol was purchased from American Radiolabeled Chemicals, Inc., St Louis, MO, USA; and [33P]orthophosphate from NEN, Boston, MA, USA. Rabbit affinity-purified antibodies to p44/p42 MAP kinases (ERK1/2) and phospho-specific (Thr 202/Tyr 204) p44/p42 MAP kinases (ERK1/2) were purchased from New England Biolabs, Inc., Beverly, MA, USA. Anti-rat mGluR5 polyclonal antibody was purchased from Upstate Biotechnology Inc., Lake Placid, NY, USA. Protein A Sepharose CL-4B beads were purchased from Amersham Pharmacia Biotech AB, Uppsala, Sweden. Phosphate-free Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Mediatech, Herndon, VA, USA, and all other media and supplements from Gibco, Gaithersburg, MD, USA. Membrane preparations used in positive controls for mGluR5 immunoblots were prepared from human embryonic kidney cell lines stably transfected to express mGluR5 by Dr Carmelo Romano (Washington University, St Louis, MO, USA).

Cell culture

Purified secondary astrocytic cultures were prepared by the method of McCarthy and de Vellis (1980) as modified by Miller et al. (1993). In brief, neocortices from 2- to 4-day-old Sprague–Dawley rat pups were dissected and dissociated in medium by trituration. The cells were centrifuged and resuspended in DMEM supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 2 mM L-glutamine, and PenStrep in tissue culture flasks and the medium was changed the following day. Cell cultures were maintained at 37°C in an atmosphere of 95% air and 5%
carbon dioxide for 6–8 days. One day after overnight shaking (280–310 r.p.m.) to remove oligodendrocytes and microglia, the cells were trypsinized and replated into poly n-lysine precoated plastic multiwell plates in DMEM with 10% FBS. After one day, the medium was replaced with DMEM and G-5 supplement (Gibco), containing epidermal growth factor (10 ng/mL), basic fibroblast growth factor (5 ng/mL), insulin (5 μg/mL) and other factors, which profoundly increases the expression of mGluR5 (Miller et al. 1995). Within 2 days, the cells were nearly confluent and resembled the stellate appearance of astrocytes in vivo. When the cultures were used in experiments 3–5 days after adding G-5 supplemented DMEM, almost no other cell morphologies were evident. Immunostaining verified that the cultures were > 95% glial fibrillary acidic protein (GFAP)-positive. The day before each experiment was conducted, the medium was removed and replaced with t-glutamine-free DMEM supplemented with PenStrep.

Treatment of astrocytes and preparation of samples for gel electrophoresis

For ERK2 phosphorylation experiments, aliquots of concentrated (100 x) BIS I stock solutions were added to triplicate wells and incubated at 37°C in an atmosphere of 95% air and 5% carbon dioxide. Aliquots of DHPG stock solutions were added at predetermined time points, and at the end of the incubation, solutions were quickly aspirated, an aliquot of cold homogenization buffer (50 mM Tris-HCl, 50 mM NaCl, 5 mM EDTA, 10 mM EGTA, 1 mM Na3VO4, 2 mM Na2P2O7·10H2O, 4 mM magnesium para-nitrophenyl phosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, and 2 μg/mL aprotinin) was added to each well, and the cells were frozen in liquid nitrogen. The cells were harvested, transferred to Eppendorf tubes, homogenized by brief sonication, and solubilized in SDS sample buffer. Protein concentrations were determined by the bicinchoninic acid assay (Pierce, Rockford, IL, USA) using bovine serum albumin as the standard.

Immunoblotting and quantitative densitometry

Samples of cell homogenates containing equal amounts of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel (12% polyacrylamide) electrophoresis (SDS-PAGE), and transferred to Immobilon-P membranes (Millipore Corporation, Bedford, MA, USA) by electroblotting. Blots were incubated for 1 h in Tris-buffered saline (TBS) and 0.1% Tween-20 (TBS-T), and then overnight at 4°C in p44/p42 MAP kinases (ERK1/2) antibody, or phosphospecific (Thr202/Tyr 204)p44/p42 MAP kinases (ERK1/2) antibody (1 : 1000). Blots were washed with TBS-T and then 5% non-fat milk in TBS-T, incubated for 1 h in horseradish peroxidase-conjugated goat anti-rabbit IgG as secondary antibody (1 : 10 000; Bio-Rad Laboratories, Hercules, CA, USA), washed again with 5% non-fat milk in TBS-T and in TBS, and processed for immunoreactivity using enhanced chemiluminescence (Amersham). Densitometry of immunoblots (Lynx densitometry) was used to quantify the changes in levels of ERK2 phosphorylation by normalizing phosphospecific ERK2 to total ERK2 detected and comparing the normalized values to basal levels (no drug) of phosphorylation, or by simply comparing levels of phosphospecific ERK2 to basal values. Values are expressed as percentage of basal.

mGluR5 immunoprecipitation and 32P incorporation and autoradiography

Immunoprecipitation of mGluR5 and [32P]orthophosphate incorporation was performed as described by Alagarsamy et al. (1999). Briefly, astrocytes in six-well plates were incubated with [32P]orthophosphate (1 mCi/well) for 3 h in glutamine- and phosphate-free DMEM at 37°C in an atmosphere of 95% air and 5% carbon dioxide. After treatment with BIS 1 (1 μM) or vehicle for 30 min, aliquots of DHPG (10 mM: 100 μL) were added for 5 min, then the solutions were withdrawn and the cells solubilized with RIPA buffer containing 0.1% SDS and protease and phosphatase inhibitors. Solubilized astrocytic cells were collected in 1.5 mL Eppendorf tubes, sonicated briefly, and centrifuged (16 000 g) at 4°C for 10 min The supernatant was transferred to Eppendorf tubes and incubated with anti-mGluR5 (4 μg/mL) overnight at 4°C. Protein A sepharose beads were added and incubated at 4°C for 3 h. Beads were pelleted and washed three times with RIPA buffer and once with 50 mM Tris-HCl (pH 7.4), then pelleted again. Gel-loading sample buffer (50 μL; 2 x) was added to the beads and incubated at room temperature for 30 min, then boiled for 2 min, and centrifuged (16 000 g) for 2 min at 4°C. An aliquot (35 μL) was taken from each sample and run on an SDS-PAGE (7.5% polyacrylamide) gel. The gel was incubated for 30 min in 1 M salicylic acid and dried for 30 min. Autoradiograms were generated from dried gels exposed to X-ray film overnight at −80°C.

Phosphoinositide hydrolysis

Phosphoinositide hydrolysis was determined by measuring the accumulation of tritiated inositol monophosphate in the presence of lithium. Astrocytes in 12-well plates were incubated for 2 days with 1 μCi [3H]myoinositol/well. The cells were then washed three times with Li–Krebs buffer (108 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 2 mM MgSO4·7H2O, 1.2 mM KH2PO4, 10 mM LiCl), then incubated in the presence of PKC inhibitors (30 min), or phorbols (10 min), or vehicle in Li–Krebs buffer at 37°C in an atmosphere of 95% air and 5% carbon dioxide. After treatment with DHPG, the solutions were aspirated and 0.75 mL of cold methanol was added to terminate the reaction. Cells were scraped, washed with 0.75 mL H2O, and transferred to tubes containing 0.75 mL chloroform. After brief sonication and vortex-mixing, aqueous and organic phases were separated by centrifugation at 4000 r.p.m. for 10 min. An 0.75-mL aliquot from the aqueous phase was added to anion exchange columns containing Dowex-1 (200–300 mesh in the formate form) for separation of [3H]inositol-containing compounds. [3H]Inositol monophosphate was eluted into scintillation vials and measured by liquid scintillation counting. Aliquots of the organic phase were dried and counted to determine total [3H]myoinositol incorporated and to calculate percentage conversion to inositol monophosphate.

Calcium fluorescence measurements

Astrocytes were plated onto cover slips, treated with DMEM/G-5, and switched to t-glutamine-free DMEM the day before the experiments. The cells were washed once in a saline buffer (135 mM NaCl, 5 mM KCl, 1 mM MgCl2, 10 mM HEPES, 25 mM d-glucose, 2 mM CaCl2, pH 7.4, plus sucrose to adjust osmolality to that of DMEM), then incubated for 30 min in 5 μM Fluo-3 AM at
37°C in an atmosphere of 95% air and 5% carbon dioxide, followed by another 30 min to de-esterify the Fluo-3. To eliminate calcium signals generated by the release of ATP and glutamate from astrocytes and activation of purinergic and ionotropic glutamate receptors, the antagonists PPADS (100 μM) and CNQX (10 μM) were added to the buffer used during the perfusion. Coverslips were placed in the perfusion chamber and after a baseline period (3 min) of perfusion with buffer, DHPG (100 μM) was applied and images were acquired every 2 s following 25 ms exposure to 450–490 nm light. Fluorescence was recorded through a band-passed filter (500–550 nm) using a Pentaxum System camera (Princeton Instruments, Inc.). Fluorescence intensity was measured in cell bodies using the Metafluor Imaging System program (Universal Imaging Corporation, Westchester, PA, USA) and expressed as F/F₀ where F₀ is the fluorescence intensity prior to DHPG treatment and F/Fₘₐₓ where Fₘₐₓ is the peak fluorescence intensity during DHPG treatment. Overall changes in calcium levels were estimated by multiplying F/F₀ or F/Fₘₐₓ by the time interval (2 s) between each measurement and accumulated for the length of time (i.e. 5 min or 30 s) of agonist application, to approximate the area under the curve.

**Statistical analysis**
Experimental data was analyzed by one- or two-way ANOVA for multiple comparisons, followed by post-hoc testing using Dunnett’s test of critical difference for comparisons of each condition to control (basal), or Tukey’s test for pair-wise comparisons. Where appropriate the Student’s t-test was used to evaluate differences between means. A p-value less than 0.05 was considered significant.

**Results**

**DHPG stimulates phosphorylation of mGluR5**
In cortical neuronal cultures, stimulation with the Group-I mGluR-selective agonist DHPG, or the mGluR5-selective agonist CHPG increases phosphorylation of the mGluR5 (Alagarsamy et al. 1999). To determine whether astrocytes, which express only mGluR5 under controlled culture conditions (Miller et al. 1995; Peavy and Conn 1998), also exhibited an agonist-induced, PKC-dependent phosphorylation of the receptor, we used a similar immunoprecipitation protocol. We stimulated cultured rat cortical astrocytes, labeled with [³²P]orthophosphate, with DHPG (100 μM, 5 min) in the presence and absence of the PKC inhibitor bisindolylmaleimide I (BIS I, 1 μM) (Toullec et al. 1991). The mGluR5 was immunoprecipitated from solubilized samples and separated by polyacrylamide gel electrophoresis, and the [³²P]orthophosphate incorporation was measured by autoradiography of the dried gel. Stimulation by DHPG caused an increase in the phosphorylation of mGluR5, which could be blocked by prior treatment (30 min) with BIS I (Fig. 1). These results were consistent with those reported for neurons (Alagarsamy et al. 1999) and demonstrated that stimulation of mGluR5 induced a PKC-mediated phosphorylation of mGluR5 in cultured rat cortical astrocytes.

**PKC inhibits DHPG-induced PI hydrolysis**
As mentioned previously, activation of PKC with phorbol esters attenuates Group-I mGluR-mediated PI hydrolysis, implicating PKC in receptor desensitization (Canonic et al. 1988; Schoepp and Johnson 1988; Godfrey and Taghavi 1990; Manzoni et al. 1990). We used phorbol esters to determine the functional consequences of prior PKC activation on mGluR5-mediated PI hydrolysis in astrocytes. After activation of PKC with PDBu (1 μM, 10 min), we stimulated [¹³H]inositol labeled astrocytes for 5 min with DHPG (100 μM), then separated inositol phosphates by ion exchange, and measured the accumulation of [¹³H]inositol monophosphate. PDBu inhibited the DHPG-induced PI hydrolysis nearly to basal levels, but the inactive analog 4α-phorbol had no effect (Fig. 2). PDBu and 4α-phorbol alone had no effect on basal levels of PI hydrolysis. These results were consistent with earlier reports for group-I mGluRs and confirmed that PKC activation caused inhibition of mGluR5-mediated PLCβ stimulation in cultured rat cortical astrocytes.

**Inhibition of PKC increases mGluR5-mediated PI hydrolysis**
The results of the previous experiment confirmed that agonist-independent activation of PKC abolished DHPG-induced PI hydrolysis. Yet, mGluR-mediated activation of PKC is more rapid and transient than phorbol-induced activation (Manzoni et al. 1990). Therefore, to examine the effects of mGluR-mediated activation of PKC on receptor...
desensitization, we measured DHPG-induced PI hydrolysis during the initial period of agonist application in the presence and absence of PKC inhibitors. We stimulated [3H]inositol labeled astrocytes with DHPG (100 \mu M, 1, 3 and 5 min) in the presence and absence of phorbol 12,13-dibutyrate (PDBu, 1 \mu M). Accumulated [3H]inositol monophosphate was separated from samples and measured by liquid scintillation counting. Prior treatment with phorbol 12,13-dibutyrate (PDBu) completely inhibited DHPG-induced PI hydrolysis. The inactive phorbol analog, 4\alpha-phorbol had no effect on the DHPG-induced response. PDBu and 4\alpha-phorbol alone had no effect on basal PI hydrolysis (basal 1.97 ± 0.22; PDBu 2.36 ± 0.22; 4\alpha-phorbol 2.24 ± 0.25). The data represent three separate experiments conducted in triplicate (n = 3, mean ± SEM; *, p < 0.05).

DHPG-induced calcium transients are affected by PKC
Stimulation of mGluR5 activates PLC\beta, resulting in formation of inositol trisphosphate (IP3), which acts to release calcium from intracellular stores. The effects of desensitization exhibited upstream on the rate of DHPG-induced PI hydrolysis would likely also be evident in calcium transients triggered by the production of IP3, and provide an additional tool for the evaluation of rapid desensitization. Therefore, to examine further the role of PKC on mGluR5-mediated responses in astrocytes we measured changes in intracellular calcium concentration by calcium fluorescence. Astrocytes labeled with the calcium fluorophore Fluo-3 were treated with PDBu or BISI, then stimulated with DHPG (100 \mu M). Images of individual cells were acquired and changes in calcium fluorescence from basal measured every 2 s over the course of the experiments. Consistent with the PI hydrolysis results, we found that activation of PKC by prior treatment of astrocytes with PDBu (1 \mu M, 10 min) diminished DHPG-induced increases in intracellular calcium to nearly basal levels (Fig. 5a,b). Inhibition of PKC with BIS I (1 \mu M) potentiated the overall DHPG-induced increase (estimated by...
the area under the curve) in intracellular calcium twofold, similar to the potentiation of PI hydrolysis in the previous experiments (Fig. 5b). Consistent with the PI hydrolysis results, treatment with PDBu or BIS I alone had no effect on basal calcium fluorescence. BIS I also altered the kinetics of the DHGP-induced calcium transients (Fig. 5a). Application of DHGP caused an immediate peak of calcium fluorescence of nearly fourfold basal (3.84 ± 0.19), declining immediately and rapidly. Over the course of a 5-min application of DHGP, relative calcium fluorescence decreased to 50% of maximum within 45 s (45 ± 6 s). In the presence of BIS I, application of DHGP caused a peak of over 4.5-fold basal (4.58 ± 0.41), which was sustained, then began to decline at a slower rate than that of DHGP alone. Treatment with BIS I increased the duration of calcium fluorescence, declining to 75% of maximum at 45 s and to 50% of maximum only after more than 2 min (140 ± 16 s) of application of DHGP. It is also interesting to note that treatment with BIS I prolonged both the time to reach maximum response after beginning the application of DHGP and the duration of the maximum response. We also conducted experiments using repeated applications of DHGP (100 μM, 30 s, 3-min intervals) in the presence and absence of BIS I (1 μM). In this case, the first application of DHGP caused a diminished response to the second application that was unaffected by the presence of BIS I. Subsequent applications showed a more diminished response to DHGP alone than DHGP in the presence of BIS I (Fig. 6). In the presence of BIS I alone, basal calcium fluorescence was not affected. It is interesting to note that even in the presence of PKC inhibitors, DHGP-induced responses exhibited desensitization, suggesting that other mechanisms are also involved in mGluR5 regulation.

Fig. 4 Protein kinase C (PKC) inhibitors potentiate (R,S)-3,5-dihydroxyphenylglycine (DHGP)-induced phosphoinositide (PI) hydrolysis in astrocytes. Cultured rat cortical astrocytes were labeled with [3H]myoinositol for 2 days, then stimulated with DHGP (100 μM) for 5 min in the presence and absence of PKC inhibitors. Prior treatment with PKC inhibitors potentiates the PI hydrolysis in response to DHGP. The inactive analog BIS V, had no effect on the DHGP-induced response. PKC inhibitors alone had no effect on basal PI hydrolysis (basal 2.63 ± 0.63; BIS I 2.33 ± 0.41; BIS V 1.93 ± 0.41; Ro-31–8220 2.32 ± 0.18). The data represent three separate experiments conducted in triplicate (n = 3, mean ± SEM; *, p < 0.05).

Fig. 5 Protein kinase C (PKC) inhibition and activation alter (R,S)-3,5-dihydroxyphenylglycine (DHGP)-induced calcium responses in astrocytes. Cultured rat cortical astrocytes were labeled with the calcium fluorophore Fluo-3 AM and then stimulated with DHGP (100 μM) for 5 min, and calcium fluorescence was measured for individual cells at 2-s intervals. (a) Representative traces of individual cells from each treatment condition. (b) Treatment with the PKC inhibitor BIS I (1 μM) potentiated calcium responses to DHGP. Treatment with PDBu (1 μM) diminished calcium responses to DHGP to near basal levels. The data represent area under the curve for averages of 24–37 cells (mean ± SEM; *, p < 0.05).

Fig. 6 Desensitization of mGluR5 is altered by inhibition of protein kinase C (PKC). Cultured rat cortical astrocytes were labeled with the calcium fluorophore Fluo-3 AM, then stimulated with (R,S)-3,5-di hydroxyphenylglycine (DHGP; 100 μM) for 30 s at 3-min intervals, and calcium fluorescence was measured at 2-s intervals for individual cells. Treatment with the PKC inhibitor BIS I (1 μM) lessened the degree of desensitization of responses to DHGP. The data represent averages of 55–65 cells (mean ± SEM; *, p < 0.05).
results of these experiments showed that mGluR5-mediated calcium responses exhibited a pattern of PKC-dependent regulation similar to the PI hydrolysis responses.

PKC inhibition has no effect on mGluR5-mediated ERK2 phosphorylation

In our evaluation mGluR5 desensitization, we also determined the role of PKC in other mGluR5-mediated signaling pathways. In addition to activating PLCβ, stimulation of mGluR5 has been shown to phosphorylate the MAP kinase ERK2 in cultured cortical astrocytes (Peavy and Conn 1998). The time course of mGluR5-mediated ERK2 phosphorylation and dephosphorylation exhibited kinetics similar to PI hydrolysis. We conducted experiments to measure the effects of PKC inhibition on DHPG-induced ERK2 phosphorylation in astrocytes using a similar strategy to the PI hydrolysis and calcium fluorescence time course experiments. Astrocytes were incubated in the presence of absence of BIS I (1 μM, 30 min), then stimulated with DHPG (100 μM) for 5–60 min, harvested and evaluated for ERK2 phosphorylation by SDS-PAGE and immunoblotting as described in Materials and methods. In contrast to the experiments measuring mGluR5-mediated PI hydrolysis and calcium transients, our results showed no effect of BIS I on the kinetics of the DHPG-induced ERK2 phosphorylation (Fig. 7). In either the presence or absence of BIS I, maximum DHPG response was achieved at 5 min of application, with almost identical rates of dephosphorylation – less than 50% at 30 min and nearly basal at 1 h. In separate experiments, similar results were observed at 1, 3 and 5 min (data not shown). These results confirm the conclusion of earlier studies in Xenopus oocyte expression systems with PKC consensus site mutants and neuronal cultures (Gereau and Heinemann 1998; Alagarsamy et al. 1999). In the oocyte studies, calcium-dependent chloride currents showed a rapid, PKC-dependent desensitization that was reversed by protein phosphatases. This was also true in cortical neurons, measuring calcium current desensitization that was lessened by PKC inhibitors (Alagarsamy et al. 1999). Similarly, in astrocytes, the degree of desensitization of DHPG-induced calcium transients was lessened by PKC inhibitors. Again these results suggest a rapid, PKC-dependent regulation of mGluR5-mediated responses, but also possibly another mechanism of desensitization that is insensitive to PKC inhibition.

In contrast to reports of PKC regulation of mGluR5 signaling through PLCβ, DHPG-induced ERK2 phosphorylation in cultured astrocytes is not regulated by PKC, although the kinetics are similar. Our previous report showed that mGluR5-mediated ERK2 phosphorylation is not dependent on the activation of PKC (Peavy and Conn 1998). We have also shown recently that other signaling components of PLCβ are not responsible for the activation of ERK2

Discussion

The results presented in this paper show that mGluR5-mediated PI hydrolysis is regulated by PKC at an early point in the signaling. In contrast, mGluR5-mediated ERK2 phosphorylation is not regulated by PKC, but perhaps is regulated by another mechanism of desensitization. This suggests that, while the two responses exhibit similar kinetics of desensitization, they have different mechanisms of regulation.

Our finding that PKC regulates mGluR5-mediated PI hydrolysis confirms earlier reports in brain slices and neuronal cultures (Canonico et al. 1988; Schoepp and Johnson 1988; Godfrey and Taghavi 1990; Manzoni et al. 1990), but supports a rapid phase in PKC-mediated desensitization. In this regard, it is interesting to note that in cultured astrocytes, longer applications (15 min–24 h) of agonist caused desensitization to a second application that was not affected by PKC inhibitors (Balazs et al. 1997). This appears to be consistent with our finding the PI hydrolysis response is not potentiated further by PKC inhibitors after the first 5 min of agonist application. After 5 min stimulation, the transient, agonist-induced activation of PKC has declined to basal levels (Manzoni et al. 1990), and no longer exerts its effects on the PLCβ signaling pathway. It is possible that another agonist-induced mechanism has begun to dominate the desensitization.

Our results with calcium also indicate a rapid, PKC-mediated regulation of mGluR5 signaling through PLCβ, and confirms the conclusions of earlier studies in Xenopus oocyte expression systems with PKC consensus site mutants and neuronal cultures (Gereau and Heinemann 1998; Alagarsamy et al. 1999). In the oocyte studies, calcium-dependent chloride currents showed a rapid, PKC-dependent desensitization that was reversed by protein phosphatases. This was also true in cortical neurons, measuring calcium current desensitization that was lessened by PKC inhibitors (Alagarsamy et al. 1999). Similarly, in astrocytes, the degree of desensitization of DHPG-induced calcium transients was lessened by PKC inhibitors. Again these results suggest a rapid, PKC-dependent regulation of mGluR5-mediated responses, but also possibly another mechanism of desensitization that is insensitive to PKC inhibition.
(Peavy et al. 2001). Taken together, these results suggest that mGluR5-mediated PLCβ activation and ERK2 phosphorylation are divergent signaling pathways that can be differentially regulated by PKC. It is possible that after the initial activation of PKC and desensitization of PLCβ signaling, both pathways are regulated by another desensitization mechanism.

There is only recent evidence to suggest that G protein coupled receptor kinases (GRK) regulate group-I mGluR signaling. In HEK293 cells that were transiently transfected to express mGluR1a, GRKs were shown to desensitize the receptor (Dale et al. 2000; Sallese et al. 2000). We saw similar results in our laboratory, with HEK293 cells that were transiently transfected to express mGluR5 (Sorensen and Conn, unpublished observations). In addition, internalization of mGluR1a, natively expressed in Purkinje cells appears to be mediated by GRK4 (Sallese et al. 2000). We have found native expression of GRK2 and GRK3 in astrocytes, which could probably play a role in the desensitization of mGluR5 responses (Sorensen and Conn, unpublished observations).

Receptor desensitization often is seen as a feedback mechanism for protection against acute or chronic overstimulation. More recently, however, the processes of desensitization and subsequent receptor internalization, resensitization and degradation have been shown to yield a multiplicity of regulatory functions and integration of signals originating from the stimulation of a single receptor subtype (Ferguson 2001). In some systems, desensitization of mGluRs has provided a differential, activity-dependent switch to inhibit signaling of one pathway while activating or potentiating a second pathway coupled to the same receptor (Herrero et al. 1998; Rodriguez-Moreno et al. 1998; Francesconi and Duvoisin 2000). This ability of a receptor subtype to couple to multiple signaling pathways under differing regulatory control confers greater complexity as well as specificity to cellular signaling events and physiological outcomes. Our research suggests that mGluR5 may also possess these signaling and regulatory properties for the PLCβ and ERK2 pathways.

Acknowledgements

The authors would like to thank Nancy Ciliax for her excellent technical support, and Dr Sudar Alagarsamy for her valuable technical advice in mGluR5 immunoprecipitation and [32P] incorporation. This work was supported by NIH/NIMH and NINDS grants.

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