Published in final edited form as:

J Neurochem. 2000 February ; 74(2): 564–573.

Mitogenic Signaling via Endogenous κ -Opioid Receptors in C6 Glioma Cells: Evidence for the Involvement of Protein Kinase C and the Mitogen-Activated Protein Kinase Signaling Cascade

Laura M. Bohn, Mariana M. Belcheva, and Carmine J. Coscia

E. A. Doisy Department of Biochemistry and Molecular Biology, St. Louis University School of Medicine, St. Louis, Missouri, U.S.A

Abstract

As reports on G protein-coupled receptor signal transduction mechanisms continue to emphasize potential differences in signaling due to relative receptor levels and cell type specificities, the need to study endogenously expressed receptors in appropriate model systems becomes increasingly important. Here we examine signal transduction mechanisms mediated by endogenous κ -opioid receptors in C6 glioma cells, an astrocytic model system. We find that the κ -opioid receptor-selective agonist U69,593 stimulates phospholipase C activity, extracellular signal-regulated kinase 1/2 phosphorylation, PYK2 phosphorylation, and DNA synthesis. U69,593-stimulated extracellular signal-regulated kinase 1/2 phosphorylation is shown to be upstream of DNA synthesis as inhibition of signaling components such as pertussis toxin-sensitive G proteins, L-type Ca^{2+} channels, phospholipase C, intracellular Ca^{2+} release, protein kinase C, and mitogen-activated protein or extracellular signal-regulated kinase blocks both of these downstream events. In addition, by overexpressing dominant-negative or sequestering mutants, we provide evidence that extracellular signal-regulated kinase 1/2 phosphorylation is Ras-dependent and transduced by $\text{G}_{\beta\gamma}$ subunits. In summary, we have delineated major features of the mechanism of the mitogenic action of an agonist of the endogenous κ -opioid receptor in C6 glioma cells.

Keywords

Opioid receptor; Opioids; Mitogen; activated protein kinase; Glioma; PYK2; Protein kinase C

The μ , κ , and δ members of the opioid receptor (OR) family are expressed by astroglial cells (Eriksson et al., 1990, 1991; Barg et al., 1991, 1993; Stiene-Martin and Hauser, 1991; Ruzicka et al., 1995; Bohn et al., 1998; Stiene-Martin et al., 1998). Opioid signaling via these G protein-coupled receptors (GPCRs) can either inhibit (Stiene-Martin and Hauser, 1991; Barg et al., 1994; Gurwell et al., 1996; Hauser et al., 1996) or stimulate (Barg et al., 1993; Gorodinsky et al., 1995) glial cell proliferation. Because astrocytes are intimately associated with neurons and can effect neuronal responses and play a role in neuronal development, regulation of astroglial growth during brain ontogeny is critical (Gasser and Hatten, 1990; Lin et al., 1993; Nedergaard, 1994; Parpura et al., 1994). Current evidence suggests that κ -OR-selective agonists activate L-type Ca^{2+} channels (Eriksson et al., 1993) and increase astroglial proliferation (Barg et al., 1993).

C6 cells have been widely used as an astrocytic model system for the study of neurotrophic actions mediated by mitogens such as endothelin (MacCumber et al., 1990; Chuang et al., 1991; Couraud et al., 1991; Lin et al., 1992; Barg et al., 1994), nerve growth factor (Kumar et al., 1990), insulin-like growth factor (Lowe et al., 1992), and basic fibroblast growth factor (Okumura et al., 1989; Luo and Miller, 1996). On treatment with the tricyclic antidepressant desipramine (DMI), both high-affinity β -endorphin and μ -OR-selective agonist binding is induced (Albouz et al., 1982; Reggiani et al., 1987; Barg et al., 1991; Bohn et al., 1998). Recently, κ -OR expression by C6 cells has been documented by RNase protection assays (L.M.B., unpublished data), RT-PCR analysis, and radioligand binding studies, which reveal a $B_{\max}$ of 75 fmol/mg of protein (Bohn et al., 1998). In addition, these cells bind the κ -selective agonist U69,593 independent of DMI pretreatment. Therefore, C6 cells are an attractive model system for the study of the signal transduction mechanisms used by endogenously expressed κ -ORs.

ORs can activate the mitogen-activated protein (MAP) kinase signaling cascade when overexpressed in Chinese hamster ovary or COS cells (Avidor-Reiss et al., 1996; Fukuda et al., 1996; Li and Chang, 1996; Belcheva et al., 1998; Ignatova et al., 1999). In vivo, chronic morphine treatment stimulates MAP kinase activity in the ventral tegmental area of rat brain (Berhow et al., 1996). A correlation between astrocyte proliferation and endothelin GPCR-mediated stimulation of the MAP kinase cascade has been demonstrated (Lazarini et al., 1996). Recently, it has been proposed that GPCR activation of the MAP kinase signaling cascade can be mediated by a putative member of the focal adhesion kinase family, PYK2 (also termed RAFTK and $CAK\beta$), in PC12 cells, which are a neuronal model system (Avraham et al., 1995; Lev et al., 1995; Soltoff, 1998; Soltoff et al., 1998).

Here we examine the signaling pathways activated by κ -opioids in C6 cells. Furthermore, we evaluate whether κ -ORs can mediate increased proliferation of C6 cells and whether activation of the MAP kinase signaling cascade is involved. We show that the κ -OR-selective agonist U69,593 stimulates proliferation of C6 cells via a pertussis toxin (PTX)-sensitive activation of phospholipase C (PLC), protein kinase C (PKC), and extracellular signal-regulated kinase (ERK). By use of interfering or dominant-negative mutants we demonstrate that ERK phosphorylation is dependent on functional $G\beta\gamma$ subunits and Ras. In addition, we also present the observation that U69,593 increases phosphorylation of PYK2.

MATERIALS AND METHODS

Reagents

U69,593 was provided by NIDA Drug Supply (Research Triangle, NC, U.S.A.). Nor-binaltorphimine (nor-BNI) was from RBI (Natick, MA, U.S.A.). Dulbecco's modified Eagle's medium (DMEM), minimal essential medium (MEM), calf serum (CS), and LIPOFECTAMINE were from Life Technologies (Grand Island, NY, U.S.A.). Fetal bovine serum (FBS) was from Harlan Bioproducts (Indianapolis, IN, U.S.A.). PTX was from List Biological Laboratories (Campbell, CA, U.S.A.). U73,122, phorbol 12-myristate 13-acetate (PMA), bisindolylmaleimide I (GFX), PD98059, and BAPTA were purchased from Calbiochem (La Jolla, CA, U.S.A.). Secondary antibodies (Abs), nifedipine, dantrolene, dimethyl sulfoxide, and other reagents were purchased from Sigma (St. Louis, MO, U.S.A.). Anti-phosphoERK1/2 (SC7383), anti-ERK1 (SC93), and anti-CD8 (SC7188) were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-Ras (R02120) was obtained from Transduction Laboratories (Lexington, KY, U.S.A.). We thank Dr. J. Baldassare (Department of Pharmacological and Physiological Sciences, St. Louis University) for the dominant-negative mutant N17Ras in pcDNAIII and Dr. S. Gutkind (National Institutes of Health) for CD8 in pcDNAI AMP and CD8- β -adrenergic receptor kinase (β ARK)-C in pcDNAIII.

Cell culture

C6 cells are known to express several growth factors as well as opioid peptides (Okumura et al., 1989; Lowe et al., 1992; Verity et al., 1998). Glucose has been shown to stimulate insulin-like growth factor expression in C6 cells (Straus and Burke, 1995). Therefore, potential paracrine and autocrine effects have been considered when studying growth regulation in C6 cells. We have carefully controlled the levels of glucose, glutamine, inositol, and serum in the growth medium as well as the number of times the cells were passaged. Because several C6 clones exist, to assure continuity and consistency throughout our experiments, we purchased cells at passage 39 from the American Type Culture Collection and maintained them according to a strict regimen. Cells were initially grown in DMEM plus 10% FBS (heat-inactivated) for two passages. At that time, medium was replaced with DMEM plus 5% CS (heat-inactivated) and then passaged an additional eight passages. Cells were used between passages 50 and 62. In each experiment, superconfluent cells were collected in phosphate-buffered saline (PBS) plus EDTA, and pellets were resuspended and plated in DMEM plus 10% FBS (150,000 or 250,000 cells per well in 12- or six-well plates, respectively, unless otherwise noted). After allowing cells to recover and adhere to the plate surface (5–7 h), medium was removed, and cells were washed with MEM. The following medium was then used to achieve optimal starvation: MEM lacking glucose, inositol, and glutamine (prepared by Washington University Tissue Culture Labs, St. Louis) with 10% MEM (GibcoBRL) to yield final concentrations of 100 mg/L glucose and 0.2 mg/L inositol (serum- and L-glutamine-free). Cells were maintained in this “low MEM” for 48 h before agonist stimulation. In all assays, agonists, antagonists, and BAPTA were delivered in glucose- and serum-free MEM. Inhibitor stocks were made in dimethyl sulfoxide and diluted in glucose- and serum-free MEM to yield <0.1% dimethyl sulfoxide per well on treatment. PTX stocks were prepared in water.

Transient transfections

C6 cells were plated in DMEM plus 5% CS at 200,000 cells per well in six-well plates. After overnight growth, wells were ~70% confluent. Cells were washed twice in MEM (Gibco-BRL) and were transfected with 1 μ g of cDNA (CD8-pcDNAI, CD8- β ARK-pcDNAI, N17Ras-pcDNAIII, or pcDNAIII) and 2 μ l of LIPOFECTAMINE (GibcoBRL) per well as described by Belcheva et al. (1998). After 14 h, transfection medium was replaced with DMEM plus 10% FBS, and cells were allowed to recover for 5–7 h. Cells were washed with MEM, and medium was replaced with “low MEM” for 48 h. ERK assays were performed as described below. In parallel samples, overexpression was verified by immunoblot analysis with anti-CD8 (CD8 was not detected in untransfected cells) or anti-Ras Abs (yields 10-fold more Ras immunoreactivity on transfection with Ras compared with vector alone).

[³H]Thymidine incorporation

Following starvation for 48 h, drugs were added to each well in 12-well plates. Antagonists and inhibitors were added at times indicated before agonist stimulation, and 0.5 μ Ci of [³H] thymidine (24 Ci/mmol; Amersham, Arlington Heights, IL, U.S.A.) per well was added 30 min after agonist. Following 24 h of agonist treatment, cells were washed twice in 5% trichloroacetic acid and then collected in 2% NaHCO₃/0.1 M NaOH (Cheng et al., 1997). [³H]Thymidine incorporation was measured by liquid scintillation counting.

Phosphoinositide (PI) turnover

Following starvation for 48 h, cells in six-well plates were labeled overnight in the same medium with 1.5 μ Ci of *myo*-[2-³H(N)]inositol (20 Ci/mmol; NEN, Boston, MA, U.S.A.) per well. PTX was added to the labeling medium for the overnight treatment. Labeling medium was replaced with “low MEM” containing 5 mM LiCl 30 min before agonist treatment. Nor-BNI was added 30 min before changing labeling medium and was added again to the fresh

LiCl medium for a total of 1 h of antagonist treatment before addition of U69,593. Following a 1-h U69,593 stimulation of PI turnover (in the presence of all inhibitors), cells were washed twice in cold PBS and collected in PBS containing EDTA with scraping. ^3H -inositol phosphate (^3H -IP_x; IP_x = inositol monophosphate + inositol bisphosphate + inositol trisphosphate) fractions were extracted in methanol/chloroform (1:1 vol/vol) and eluted from Bio-Rad AG1-X8 columns with 1 M ammonium formate in 0.1 M formic acid as described (Barg et al., 1994). Relative ^3H -IP_x levels were measured by scintillation counting and calculated as percentages of unstimulated controls.

ERK assays

Following starvation for 48 h, cells in six-well plates were treated as indicated. Antagonists and inhibitors were added to the medium 1 h and 30 min before stimulation with agonist, respectively. After the indicated stimulation period, medium was removed, and plates were washed twice with cold PBS. Cell lysates were collected in lysing buffer (20 mM HEPES, 10 mM EGTA, 40 mM β -glycerophosphate, 2.5 mM MgCl₂, 2 mM sodium vanadate, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 20 $\mu\text{g}/\text{ml}$ aprotinin, and 20 $\mu\text{g}/\text{ml}$ leupeptin, pH 7.5) and cleared by centrifugation (10 min, 15,000 g). Protein assays were performed using the Bradford reagent (Bio-Rad, Hercules, CA, U.S.A.), and lysates were then denatured in gel loading buffer by boiling. Equivalent amounts of protein were loaded per lane (10–15 μg) on 10% sodium dodecyl sulfate-polyacrylamide mini-gels. Following electrophoretic transfer to Immobilon-P polyvinylidene difluoride membranes (Millipore, Bedford, MA, U.S.A.), membranes were blocked with 1% bovine serum albumin plus 1% nonfat dry milk in Tris-buffered saline with Tween [10 mM Tris base (pH 8.0), 150 mM NaCl, and 0.5% Tween-20], and immunoblots were performed using anti-phosphoERK1/2 (1:1,000 dilution) and peroxidase-conjugated anti-mouse secondary Ab (1:7,000). Bands were detected by chemiluminescence and exposure to X-Omat diagnostic film (Eastman Kodak, Rochester, NY, U.S.A.). For assurance of equivalent total ERK protein per lane, blots were stripped [at 50°C for 30 min in 62.5 mM Tris (pH 6.8), 0.1 M β -mercaptoethanol, and 2% (wt/vol) SDS] and exposed to Abs for ERK1 (1:1,000 dilution). Band intensities were determined by densitometric analysis using NIH Image software. To corroborate the data gained with the immunoblotting methodology, endogenous κ -OR-mediated ERK activation was also measured by an autoradiographic kinase assay (M.M.B., unpublished data). In this “in-gel” assay, ERK activity (as expressed by the degree of phosphorylation of myelin basic protein) was quantified using a PhosphorImager (Molecular Dynamics) and Image Quant software. In addition, the two- to threefold phosphorylation of ERK observed is comparable to the activation of ERK seen for overexpressed κ -ORs in COS-7 and human embryonic kidney (HEK) 293 cells (Belcheva et al., 1998; Ignatova et al., 1999).

PYK2 assays

C6 cells were plated in DMEM plus 10% FCS at 800,000 cells per 100-mm-diameter plate. Cells were allowed to adhere to plates for 5 h, and then medium was changed to “low MEM.” After 48 h in “low MEM,” U69,593 (10 nM) was added directly to the medium for the 3-min treatment. Two equally confluent, 100-mm-diameter plates were used for each immunoprecipitation. This method was followed as carefully described by Soltoff (1998) with minor changes. Treatment with drugs was terminated by washing cells twice in ice-cold buffered saline (TNE; 137 mM NaCl, 20 mM Tris base, 1 mM EDTA, and 0.2 mM sodium vanadate, pH 7.5), and then cells were immediately lysed in lysis buffer [137 mM NaCl, 20 mM Tris base (pH 7.5), 1 mM EGTA, 1 mM EDTA, 10% (vol/vol) glycerol, and 1% (vol/vol) Nonidet P-40] with the following protease and phosphatase inhibitors: 1 mM sodium vanadate, 1 mM ZnCl₂, 4.5 mM sodium pyrophosphate, 2 mg/ml NaF, 2 mg/ml β -glycerophosphate, 10 $\mu\text{g}/\text{ml}$ pepstatin, 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ aprotinin, and 1 mM phenylmethylsulfonyl fluoride. Lysates were cleared by centrifugation at 15,000 g for 5 min and then were incubated

for 4 h at 4°C in the presence of protein A-Sepharose (4 mg/ml) and anti-phosphotyrosine Ab (5 μ l). Protein A-Sepharose-Ab-protein complexes were collected and washed as described by Soltoff (1998) and then disrupted by boiling in gel-loading buffer. Proteins were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by transfer to Immobilon-P membranes. Membranes were blocked with 1% bovine serum albumin plus 1% nonfat dry milk in Tris-buffered saline with Tween and then blotted with anti-PYK2 Ab (1:200) and per-oxidase-conjugated anti-mouse secondary Ab (1:7,000).

Statistical analysis

Statistical determinations were made either by one-way ANOVA followed by Dunnett's post hoc test or by Student's *t* test analysis where indicated using GraphPad Prism software (version 2.01; GraphPad Software).

RESULTS

Inhibition of κ -OR agonist-stimulated DNA synthesis in C6 glioma cells

The κ -OR-selective agonist U69,593 stimulates DNA synthesis in a concentration-dependent manner with an of 1.03 nM (Fig. 1A). The degree of U69,593 EC₅₀ stimulation of DNA synthesis is comparable to that seen with endothelin-1 and basic fibroblast growth factor in this cell line (MacCumber et al., 1990; Chuang et al., 1991; Couraud et al., 1991; Lin et al., 1992; Barg et al., 1994; Luo and Miller, 1996) and is prevented by the κ -selective antagonist nor-BNI (Fig. 1B). Selective inhibitors directed against potential second messengers were used at concentrations close to their corresponding IC₅₀ values to block signaling components activated in the κ -opioid stimulation of DNA synthesis (Fig. 1B). PTX abolishes U69,593-induced [³H]thymidine incorporation, suggesting the involvement of G_{i/o} proteins. The activity of L-type Ca²⁺ channels proves to be essential in relaying κ -OR signaling as nifedipine, a selective inhibitor of L-type Ca²⁺ channels, blocks U69,593 stimulation of DNA synthesis. The PLC inhibitor U73122 also blocks κ -opioid stimulation of [³H]thymidine incorporation. Activation of PLC leads to increases in inositol 1,4,5-trisphosphate and subsequent increases in intracellular Ca²⁺ levels. Using dantrolene to block the release of intracellular Ca²⁺ stores and BAPTA to chelate intracellular Ca²⁺, we inhibit U69,593-stimulated [³H]thymidine incorporation, suggesting that increases in intracellular Ca²⁺ levels are essential for U69,593 stimulation of DNA synthesis (Fig. 1B).

Inhibition of κ -opioid-stimulated PI turnover

The direct activation of PLC was assayed by measuring PI turnover (Fig. 2). U69,593 stimulates PI hydrolysis, and this action can be blocked by nor-BNI, PTX, and U73,122. Addition of nifedipine modestly attenuates U69,593-induced PI turnover, indicating that the initial influx of Ca²⁺ via L-type Ca²⁺ channels may be necessary for full PLC activation (Smart et al., 1995). Nifedipine, nor-BNI, U73,122, BAPTA, and PTX when administered alone do not affect basal levels of growth or PI turnover (data not shown).

Implication of PKC stimulation in κ -opioid mitogenic signaling

The activation of PLC leads to elevated diacylglycerol levels and increases in intracellular Ca²⁺ levels, which result in the stimulation of PKC (Nishizuka, 1984). Overnight treatment with 1 μ M PMA leads to degradation of conventional PKC isoforms in C6 cells (Chen, 1993), and this treatment abolishes U69,593-stimulated DNA synthesis (Fig. 3). Because PMA, under these conditions, decreases basal levels of DNA synthesis by ~15% (data not shown), another means of blocking PKC was used. The PKC-selective inhibitor GFX also inhibits U69,593-stimulated DNA synthesis (Fig. 3). To demonstrate that direct PKC stimulation could promote proliferation, C6 cells were treated with 100 pM PMA. The direct stimulation of PKC

by phorbol ester results in increases of [^3H]thymidine incorporation ($130 \pm 8\%$ over nontreated control, $n = 3$) similar to levels seen for U69,593 treatment. PMA stimulation of DNA synthesis is completely blocked by GFX ($96 \pm 8\%$ of nontreated control, $n = 3$); GFX when used alone did not affect basal levels of thymidine incorporation ($101 \pm 6\%$ of nontreated control, $n = 3$).

PKC has been shown to stimulate Raf (Kolch et al., 1993), thereby implicating a potential route for GPCR-mediated mitogenic signaling (reviewed by Gutkind, 1998). Because the U69,593 effect on proliferation is dependent on PKC activation, we used an inhibitor of ERK kinase (MEK), PD98059, to ascertain the involvement of ERK in this signaling cascade. PD98059 completely blocks U69,593 stimulation of DNA synthesis (Fig. 3), suggesting that the mitogenic signal from the κ -OR progresses via an MEK/ERK-dependent pathway.

κ -Opioid stimulates ERK1/2 phosphorylation

PD98059 inhibition of U69,593-stimulated DNA synthesis suggests that ERK may be a downstream effector in κ -OR signaling. Therefore, the effect of U69,593 on ERK1/2 phosphorylation was examined by western blot analysis using monoclonal Abs specific to phosphorylated ERK1/2. U69,593 (5 nM) stimulates a time-dependent phosphorylation of ERK1/2 that is first apparent 1 min after treatment (Fig. 4A). ERK1/2 phosphorylation plateaus between 5 and 30 min and decreases after a 1-h agonist treatment, possibly representing desensitization of the receptor. During a 5-min exposure, U69,593 stimulates ERK1/2 phosphorylation in a concentration-dependent manner (Fig. 4B); phosphorylation levels plateau at 1 nM U69,593, which corresponds to the EC_{50} seen for U69,593 stimulation of [^3H]thymidine incorporation (Fig. 1A). On the basis of the above data, we propose a model for inducing proliferation that involves increased ERK1/2 phosphorylation initiated by stimulation of the κ -OR. Accordingly, we found that U69,593-induced ERK1/2 phosphorylation is prevented by the same inhibitors that also block U69,593 stimulation of DNA synthesis: nor-BNI, PTX, nifedipine, U73,122, dantrolene, BAPTA, PMA (1 μM , overnight), GFX, and PD98059 (Fig. 5). These findings suggest that similar signaling components required for increased cell proliferation may also be necessary for ERK1/2 activation, implicating this MAP kinase upstream of increased DNA synthesis in response to U69,593. Stimulation of ERK1/2 by basic fibroblast growth factor (2.4 ± 0.02 -fold phosphorylation over control, $n = 3$), which acts via a receptor tyrosine kinase, is not inhibited by nor-BNI, PTX, nifedipine, U73,122, dantrolene, or BAPTA at the same inhibitor levels used to block κ -opioid action, indicating that the inhibitors are acting on components of the GPCR signaling cascade in a selective manner. As expected, PD98059 completely blocks basic fibroblast growth factor phosphorylation of ERK1/2 (data not shown).

Role of $\text{G}\beta\gamma$ in κ -OR-mediated signaling

U69,593 stimulation of cell proliferation, PI turnover, and ERK1/2 phosphorylation is PTX-sensitive, implying sub-transduction by heterotrimeric $\text{G}_{i/o}$ proteins. The $\beta\gamma$ units of this family of G proteins stimulate PLC (Smrcka and Sternweis, 1993; Wu et al., 1993; Stehno-Bittel et al., 1995) in a PTX-sensitive fashion (Dorn et al., 1997). Because U69,593-stimulated ERK1/2 phosphorylation requires PLC activity and is PTX-sensitive (Fig. 5), we evaluated the contribution of $\text{G}\beta\gamma$ in U69,593 phosphorylation of ERK1/2 by transiently transfecting C6 cells with CD8- $\beta\text{ARK-C}$ (Fig. 6). The C-terminal domain of βARK sequesters $\text{G}\beta\gamma$ subunits; overexpression of CD8- $\beta\text{ARK-C}$ has been shown to sequester $\text{G}\beta\gamma$ subunits, thereby selectively preventing their involvement in activating second messengers (Crespo et al., 1995; van Biesen et al., 1995). Overexpression of CD8- $\beta\text{ARK-C}$ attenuates U69,593 stimulation of ERK1/2 phosphorylation, whereas transfection of the vector +CD8 alone has no significant effect (Fig. 6). This observation suggests that $\text{G}\beta\gamma$ subunits are required in the phosphorylation of ERK1/2 via the κ -OR.

Ras dependency of κ -opioid phosphorylation of ERK1/2

Signal transduction mediated via $G_{i/o}$ and G_q that results in the phosphorylation of ERK1/2 has been shown to require Ras activation (Pace et al., 1995; Eguchi et al., 1996; Della Rocca et al., 1997). Previously, it was demonstrated that Ras is essential for ERK1/2 activation in COS-7 cells expressing μ -, δ -, and κ -ORs (Belcheva et al., 1998). Our data indicate that κ -OR-mediated phosphorylation of ERK1/2 in C6 cells involves activation of PKC (Fig. 5B). Direct stimulation of PKC with acute PMA (100 μ M, 3 min) treatment stimulates ERK1/2 phosphorylation in C6 cells, and this activation is blocked with GFX or PD98059 (data not shown). It was previously shown in PC12 cells (a neuronal cell model) that phorbol ester activation of PKC stimulates ERK1/2 in a Ras-dependent manner (Thomas et al., 1992). To evaluate the involvement of Ras in endogenous κ -OR signaling, we transiently transfected a dominant-negative mutant of Ras, N17Ras, into C6 cells. The N17Ras mutant prevents the activation of Raf and thereby blocks the downstream activation of ERK (de Vries-Smits et al., 1992; Wood et al., 1992; Ming et al., 1994; Hawes et al., 1995; van Biesen et al., 1996; Marais et al., 1998). When overexpressed in C6 cells, N17Ras prevents U69,593 phosphorylation of ERK1/2, suggesting that a Ras-dependent pathway is involved in κ -OR signaling (Fig. 7).

U69,593 stimulates PYK2 phosphorylation

Mechanisms by which GPCRs activate the MAP kinase signaling cascade may involve interactions with Src family tyrosine kinases (Gutkind, 1998), and such interactions may be mediated by PYK2, which is a member of the focal adhesion kinase family (Avraham et al., 1995). In PC12 and HEK293 cells, it has been proposed that G_{α_q} and $G_{\beta\gamma}$ activate PLC β , resulting in elevated intracellular Ca^{2+} and activated PKC, which can lead to tyrosine phosphorylation of PYK2 (Lev et al., 1995; Dikic et al., 1996; Della Rocca et al., 1997; Soltoff, 1998). PYK2, in turn, activates tyrosine kinases previously implicated in mediating neuronal ERK1/2 activation by GPCRs (Lev et al., 1995). To assess the role of PYK2 in κ -OR glial signaling, we measured PYK2 tyrosine phosphorylation in C6 cells after U69,593 treatment (Fig. 8). Because maximal ERK1/2 phosphorylation was achieved after a 5-min U69,593 treatment, we assayed for PYK2 phosphorylation after treating the cells with 10 nM U69,593 for 3 min. In these experiments, tyrosine-phosphorylated proteins were immunoprecipitated with anti-phosphotyrosine Ab. The immunoprecipitates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and then tyrosine-phosphorylated PYK2 was detected by western blot analysis using a monoclonal anti-PYK2 Ab. Accordingly, we found that U69,593 elicits a sixfold increase in PYK2 tyrosine phosphorylation over basal levels (Fig. 8). In further support of the involvement of a tyrosine kinase-dependent pathway, pretreatment with genistein (50 μ M, 30 min before U69,593 as described in Fig. 4) prevented U69,593 phosphorylation of ERK ($92 \pm 10\%$ of untreated controls, $n = 5$).

DISCUSSION

In this study we delineate a mechanism of a potent mitogenic action mediated by endogenous κ -ORs in C6 glioma cells. As demonstrated by the κ -OR antagonist blockade of signaling, the κ -OR-selective agonist U69,593 is acting via κ -ORs to stimulate DNA synthesis, PI turnover, and ERK1/2 phosphorylation (Figs. 1, 2, and 5). The $\beta\gamma$ subunit of a PTX-sensitive G protein has been implicated as transducer in this signaling pathway (Fig. 6). PKC appears to be a primary effector in κ -opioid signaling as down-regulation or direct inhibition of this kinase blocks downstream signaling (Figs. 3 and 5). In turn, PKC may converge with MAP kinase signaling cascade via PYK2 (Fig. 8); the site of this convergence appears to be upstream of Ras. This is supported by the observation that the same selective inhibitors block both κ -OR-mediated ERK1/2 phosphorylation and DNA synthesis (Figs. 1, 3, and 5) and is further shown by using a dominant-negative mutant of Ras transfected in C6 cells to attenuate κ -opioid phosphorylation of ERK (Fig. 7).

In a previous study from this laboratory, κ -OR agonist stimulation of DNA synthesis in mixed glial cell cultures was shown to be PTX-sensitive as well (Barg et al., 1993). Evidence for the involvement of PI turnover in the κ -opioid neurotrophic actions was also gained in the same study using primary neural cultures. Collectively, our preliminary investigation and the results presented in this report provide several lines of evidence to suggest that a stimulation of PI turnover may initiate mitogenic signal transduction. PTX-sensitive GPCR-mediated stimulation of PLC can be transduced by $\beta\gamma$ subunits of $G_{i/o}$, which was demonstrated by overexpression of β ARK-C (Crespo et al., 1995; van Biesen et al., 1995). Here, the transfection of CD8- β ARK-C prevented ERK phosphorylation by U69,593 (Fig. 6), implicating the $G_{i/o}$ $\beta\gamma$ subunits in this signaling pathway. However, there remains the potential that sequestration of $\beta\gamma$ subunits could possibly interfere with the functional coupling of receptors and G proteins. PI turnover (Fig. 2) and ERK1/2 phosphorylation (Fig. 5) induced by U69,593 were also inhibited by PTX, supporting the interpretation that these components are involved in a common signaling pathway. Similarly, the abolishment of κ -opioid-induced PI turnover, ERK1/2 phosphorylation, and DNA synthesis by the PLC inhibitor U73,122 reinforces the concept that PLC is an essential component of mitogenic signaling. Blockade of κ -opioid-induced ERK phosphorylation and DNA synthesis by the PKC inhibitor GFX and the MEK inhibitor PD98059 as well as by down-regulation of PKC is also consistent with this theory. In addition, PKC activation is associated with elevated rates of glioma proliferation (reviewed by Bredel and Pollack, 1997; Feldkamp et al., 1997), and opioid-induced intracellular Ca^{2+} mobilization correlates with their neurotrophic effects in C6 cells (Barg et al., 1994). Taken together, this body of interlocking evidence strongly supports a sequential signaling pathway involving PLC, PKC, Ras, and ERK.

κ -ORs were previously shown to couple to L-type Ca^{2+} channels in primary astrocytic cultures (Eriksson et al., 1991). In SH-SY5Y cells, agonist binding to μ -ORs was shown to stimulate PLC, which proved to be dependent on Ca^{2+} influx as pretreatment with nifedipine abolished the ability of μ -opioid agonist to stimulate PI turnover (Smart et al., 1995). Nifedipine treatment resulted in significant attenuation of U69,593-stimulated PLC activity, as well as a complete inhibition of opioid-stimulated ERK1/2 phosphorylation and DNA synthesis in C6 cells (Figs. 1, 2, and 5). Activation of PLC and subsequent increases in intracellular Ca^{2+} levels proved to be crucial for κ -opioid phosphorylation of ERK and proliferation (Figs. 1 and 5). Thus, κ -opioid-induced Ca^{2+} mobilization plays an integral role in the mitogenic mechanism.

In C6 cells, the overexpression of N17Ras prevents U69,593 stimulation of ERK1/2 phosphorylation, suggesting that Ras is involved in κ -OR mitogenic signaling (Fig. 7). The PTX-sensitive stimulation of DNA synthesis via activation of thrombin or LPA receptors in hamster lung fibroblasts or Rat-1 cells, respectively, has been shown to involve Ras activation (van Corven et al., 1993). GPCR activation of ERK1/2 via a Ras-dependent signaling cascade as shown in PC12, COS-7, Chinese hamster ovary-K1, Rat-1, HEK293, and smooth muscle cells (Lev et al., 1995; Pace et al., 1995; Avidor-Reiss et al., 1996; Eguchi et al., 1996; Della Rocca et al., 1997; Belcheva et al., 1998) appears then to represent a predominant mechanism for generating a mitogenic response via these receptors.

Because direct activation of PKC with phorbol esters stimulated mitogenic signaling via Ras in PC12 cells, a neuronal model cell line (Thomas et al., 1992), the importance of PYK2 in C6 cells was also considered. PYK2 is abundant in the CNS, and its function has been investigated primarily in PC12 cells (Avraham et al., 1995; Lev et al., 1995). Recently its occurrence and involvement in mitogenic signaling have also been reported in primary astrocytes (Cazaubon et al., 1997). Nevertheless, its possible involvement in relaying opioid responses in glial cells had not been investigated. Cellular responses to κ -OR stimulation include G protein-mediated increases in intracellular free $[Ca^{2+}]$ and activated PKC; both events have been shown to stimulate PYK2 phosphorylation and subsequently activate the MAP kinase signaling cascade

(Lev et al., 1995; Dikic et al., 1996; Soltoff, 1998; Soltoff et al., 1998). In C6 cells, U69,593 elicited a sixfold increase in PYK2 tyrosine phosphorylation over basal levels (Fig. 8). PYK2 may represent a point of convergence between the GPCR and receptor tyrosine kinase/MAP kinase pathways in these cells, and studies are underway to test this possibility. The robust increase in PYK2 phosphorylation was detected in 3 min, whereas that of ERK was maximal around 5 min. Maximal stimulation of PI turnover by U69,593 occurred in 10 min (Bohn et al., 2000), and thymidine incorporation into DNA takes hours to detect. Thus, the U69,593-induced changes are sequential in a fashion consistent with our working model of the signaling pathway. In summary, our data indicate that κ -opioid signaling via $G\beta\gamma$ units, Ca^{2+} , PLC, and PKC initiates Ras-dependent mitogenesis in the astrocytic C6 cell line.

Acknowledgements

This work was supported in part by grants DA05412 (to C.J.C.) and DA05766-03 (to L.M.B.) from the National Institutes of Health. We thank Dr. John A. Corbett for advice and critical reading of the manuscript.

Abbreviations used

Ab	antibody
βARK	β -adrenergic receptor kinase
CS	calf serum
DMEM	Dulbecco's modified Eagle's medium
DMI	desipramine
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
GFX	bisindolylmaleimide I
GPCR	G protein-coupled receptors
HEK	human embryonic kidney
IP	inositol phosphate
MAP	mitogen-activated protein
MEK	extracellular signal-regulated kinase kinase
MEM	

	minimal essential medium
nor-BNI	nor-binalthorphan
OR	opioid receptor
PBS	phosphate-buffered saline
PI	phosphoinositide
PLC	phospholipase C
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PTX	pertussis toxin

References

- Albouz S, Tocque B, Hauw JJ, Boutry JM, LeSaux F, Bourdoin R, Baumann N. Tricyclic antidepressant desipramine induces stereospecific opiate binding and lipid modification in rat glioma C6 cells. *Life Sci* 1982;31:2549–2554. [PubMed: 6296584]
- Avidor-Reiss T, Nevo I, Levy R, Pfeuffer T, Vogel Z. Chronic opioid treatment induces adenylyl cyclase V superactivation. *J Biol Chem* 1996;271:21309–21315. [PubMed: 8702909]
- Avraham S, London R, Fu Y, Ota S, Hiregowdara D, Li J, Jiang S, Pasztor LM, White RA, Groopman JE, Avraham H. Identification and characterization of a novel related adhesion focal tyrosine kinase (RAFTK) from megakaryocytes and brain. *J Biol Chem* 1995;270:27742–27751. [PubMed: 7499242]
- Barg J, Belcheva M, Bem WT, Lambourne B, McLachlan JA, Tolman KC, Johnson FE, Coscia CJ. Desipramine modulation of sigma and opioid peptide receptor expression in glial cells. *Peptides* 1991;12:845–849. [PubMed: 1664948]
- Barg J, Belcheva MM, Rowiński J, Coscia CJ. κ -Opioid agonist modulation of [3 H]thymidine incorporation into DNA: evidence for the involvement of pertussis toxin-sensitive G protein-coupled phosphoinositide turnover. *J Neurochem* 1993;60:1505–1511. [PubMed: 8384252]
- Barg J, Belcheva MM, Levy R, Saya D, McHale RJ, Johnson FE, Coscia CJ, Vogel Z. Opioids inhibit endothelin-mediated DNA synthesis, phosphatidylinositol turnover and Ca^{2+} mobilization in rat C6 glioma cells. *J Neurosci* 1994;14:5858–5864. [PubMed: 7931548]
- Belcheva MM, Vogel Z, Ignatova E, Avidor-Reiss T, Zippel R, Levy R, Young EC, Barg J, Coscia CJ. Opioid modulation of extracellular signal-regulated protein kinase activity is Ras-dependent and involves $\text{G}_{\beta\gamma}$ subunits. *J Neurochem* 1998;70:635–645. [PubMed: 9453557]
- Berhow MT, Hiroi N, Nestler EJ. Regulation of ERK (extracellular signal regulated kinase), part of the neurotrophin signal transduction cascade, in the rat mesolimbic dopamine system by chronic exposure to morphine or cocaine. *J Neurosci* 1996;16:4707–4715. [PubMed: 8764658]
- Bohn LM, Belcheva MM, Coscia CJ. Evidence for κ - and μ -opioid receptor expression in C6 glioma cells. *J Neurochem* 1998;70:1819–1825. [PubMed: 9572265]
- Bohn LM, Belcheva MM, Coscia CJ. μ -Opioid agonist inhibition of κ -opioid receptor-stimulated extracellular signal-regulated kinase phosphorylation is dynamin-dependent in C6 glioma cells. *J Neurochem* 2000;74:574–581. [PubMed: 10646508]

- Bredel M, Pollack IF. The role of protein kinase C (PKC) in the evolution and proliferation of malignant gliomas, and the application of PKC inhibition as a novel approach to anti-glioma therapy. *Acta Neurochir (Wien)* 1997;139:1000–1013. [PubMed: 9442212]
- Cazaubon S, Chaverot N, Romero IA, Girault JA, Adamson P, Strosberg AD, Couraud PO. Growth factor activity of endothelin-1 in primary astrocytes mediated by adhesion-dependent and -independent pathways. *J Neurosci* 1997;17:6203–6212. [PubMed: 9236231]
- Chen CC. Protein kinase C α , δ , ϵ , and ζ in C6 glioma cells. TPA induces translocation and down-regulation of conventional and new PKC isoforms but not atypical PKC ζ . *FEBS Lett* 1993;332:169–173. [PubMed: 8405436]
- Cheng J, Weber JD, Baldassare JJ, Raben DM. Ablation of Go α -subunit results in a transformed phenotype and constitutively active phosphatidylcholine-specific phospholipase C. *J Biol Chem* 1997;272:17312–17319. [PubMed: 9211868]
- Chuang D-M, Lin W-W, Lee CY. Endothelin-induced activation of phosphoinositide turnover, calcium mobilization, and transmitter release in cultured neurons and neurally related cell types. *J Cardiovasc Pharmacol* 1991;17 (Suppl):S85. [PubMed: 1725440]
- Couraud PO, Durieu-Trautmann O, Nguyen DL, Marin P, Glibert F, Strosberg AD. Functional endothelin-1 receptors in rat astrocytoma C6. *Eur J Pharmacol* 1991;206:191–198. [PubMed: 1649761]
- Crespo P, Cachero TG, Xu N, Gutkind JS. Dual effect of beta-adrenergic receptors on mitogen-activated protein kinase. Evidence for a beta gamma-dependent activation and a G α s-cAMP-mediated inhibition. *J Biol Chem* 1995;270:25259–25264. [PubMed: 7559665]
- Della Rocca GJ, van Biesen T, Daaka Y, Luttrell DK, Luttrell LM, Lefkowitz RJ. Ras-dependent mitogen-activated protein kinase activation by G protein-coupled receptors. Convergence of Gi- and Gq-mediated pathways on calcium/calmodulin, PYK2, and Src kinase. *J Biol Chem* 1997;272:19125–19132. [PubMed: 9235901]
- de Vries-Smits AM, Burgering BM, Leevers SJ, Marshall CJ, Bos JL. Involvement of p21ras in activation of extracellular signal-regulated kinase 2. *Nature* 1992;357:602–604. [PubMed: 1608472]
- Dikic I, Tokiwa G, Lev S, Courtneidge SA, Schlessinger J. A role for Pyk2 and Src in linking G-protein-coupled receptors with MAP kinase activation. *Nature* 1996;383:547–550. [PubMed: 8849729]
- Dorn GW 2nd, Oswald KJ, McCluskey TS, Kuhel DG, Liggett SB. Alpha 2A-adrenergic receptor stimulated calcium release is transduced by Gi-associated G(beta gamma)-mediated activation of phospholipase C. *Biochemistry* 1997;36:6415–6423. [PubMed: 9174358]
- Eguchi S, Matsumoto T, Motley ED, Utsunomiya H, Inagami T. Identification of an essential signaling cascade for mitogen-activated protein kinase activation by angiotensin II in cultured rat vascular smooth muscle cells. Possible requirement of Gq-mediated p21ras activation coupled to a Ca^{2+} /calmodulin-sensitive tyrosine kinase. *J Biol Chem* 1996;271:14169–14175. [PubMed: 8662912]
- Eriksson PS, Hansson E, Ronnback L. δ and κ opiate receptors in primary astroglial cultures from rat cerebral cortex. *Neurochem Res* 1990;15:1123–1126. [PubMed: 1982460]
- Eriksson PS, Hansson E, Ronnback L. Mu and delta opiate receptors in neuronal and astroglial primary cultures from various regions of the brain—coupling with adenylate cyclase, localisation on the same neurones and association with dopamine (D1) receptor adenylate cyclase. *Neuropharmacology* 1991;30:1233–1239. [PubMed: 1663598]
- Eriksson PS, Nilsson M, Wagberg M, Hansson E, Ronnback L. Kappa-opioid receptors on astrocytes stimulate L-type Ca^{2+} channels. *Neuroscience* 1993;54:401–407. [PubMed: 8393154]
- Feldkamp MM, Lau N, Guha A. Signal transduction pathways and their relevance in human astrocytomas. *J Neurooncol* 1997;35:223–248. [PubMed: 9440022]
- Fukuda K, Kato S, Morikawa H, Shoda T, Mori K. Functional coupling of the δ -, μ -, and κ -opioid receptors to mitogen-activated protein kinase and arachidonate release in Chinese hamster ovary cells. *J Neurochem* 1996;67:1309–1316. [PubMed: 8752140]
- Gasser UE, Hatten ME. Neuron–glia interactions of rat hippocampal cells in vitro: glial-guided neuronal migration and neuronal regulation of glial differentiation. *J Neurosci* 1990;10:1276–1285. [PubMed: 2329376]
- Gorodinsky A, Barg J, Belcheva MM, Levy R, McHale RJ, Vogel Z, Coscia CJ. Dynorphins modulate DNA synthesis in fetal brain cell aggregates. *J Neurochem* 1995;65:1481–1486. [PubMed: 7561841]

- Gurwell JA, Duncan MJ, Maderspach K, Stiene-Martin A, Elde RP, Hauser KF. Kappa-opioid receptor expression defines a phenotypically distinct subpopulation of astroglia: relationship to Ca^{2+} mobilization, development, and the antiproliferative effect of opioids. *Brain Res* 1996;737:175–187. [PubMed: 8930364]
- Gutkind JS. The pathways connecting G protein-coupled receptors to the nucleus through divergent mitogen-activated protein kinase cascades. *J Biol Chem* 1998;273:1839–1842. [PubMed: 9442012]
- Hauser KF, Stiene-Martin A, Mattson MP, Elde RP, Ryan SE, Godleske CC. Mu-opioid receptor-induced Ca^{2+} mobilization and astroglial development: morphine inhibits DNA synthesis and stimulates cellular hypertrophy through a $\text{Ca}(2+)$ -dependent mechanism. *Brain Res* 1996;720:191–203. [PubMed: 8782912]
- Hawes BE, van Biesen T, Koch WJ, Luttrell LM, Lefkowitz RJ. Distinct pathways of Gi- and Gq-mediated mitogen-activated protein kinase activation. *J Biol Chem* 1995;270:17148–17153. [PubMed: 7615510]
- Ignatova EG, Belcheva MM, Bohn LM, Neuman MC, Coscia CJ. Requirement of receptor internalization for opioid stimulation of mitogen-activated protein (MAP) kinase; biochemical and immunofluorescence confocal microscopic evidence. *J Neurosci* 1999;19:56–63. [PubMed: 9870938]
- Kolch W, Heidecker G, Kochs G, Hummel R, Vahidi H, Mischak H, Finkenzeller G, Marme D, Rapp UR. Protein kinase C alpha activates RAF-1 by direct phosphorylation. *Nature* 1993;36:249–252. [PubMed: 8321321]
- Kumar S, Huber J, Pena LA, Perez-Polo JR, Werrbach-Perez K, de Vellis J. Characterization of functional nerve growth factor-receptors in a CNS glial cell line: monoclonal antibody 217c recognizes the nerve growth factor-receptor on C6 glioma cells. *J Neurosci Res* 1990;27:408–417. [PubMed: 1965839]
- Lazarini F, Strosberg AD, Couraud PO, Cazaubon SM. Coupling of ET_B endothelin receptor to mitogen-activated protein kinase stimulation and DNA synthesis in primary cultures of rat astrocytes. *J Neurochem* 1996;66:459–465. [PubMed: 8592114]
- Lev S, Moreno H, Martinez R, Canoll P, Peles E, Musacchio JM, Plowman GD, Rudy B, Schlessinger J. Protein tyrosine kinase PYK2 involved in $\text{Ca}(2+)$ -induced regulation of ion channel and MAP kinase functions. *Nature* 1995;376:737–745. [PubMed: 7544443]
- Li L-Y, Chang KJ. The stimulatory effect of opioids on mitogen-activated protein kinase in Chinese hamster ovary cells transfected to express mu-opioid receptors. *Mol Pharmacol* 1996;50:599–602. [PubMed: 8794899]
- Lin LF, Doherty DH, Lile JD, Bektesh S, Collins F. GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science* 1993;260:1130–1132. [PubMed: 8493557]
- Lin W-W, Kiang J, Chuang DM. Pharmacological characterization of endothelin-stimulated phosphoinositide breakdown and cytosolic free Ca^{2+} rise in rat C6 glioma cells. *J Neurosci* 1992;12:1077–1085. [PubMed: 1312133]
- Lowe WL Jr, Meyer T, Karpen CW, Lorentzen LR. Regulation of insulin-like growth factor I production in rat C6 glioma cells: possible role as an autocrine/paracrine growth factor. *Endocrinology* 1992;130:2683–2691. [PubMed: 1572288]
- Luo J, Miller MW. Ethanol inhibits basic fibroblast growth factor-mediated proliferation of C6 astrocytoma cells. *J Neurochem* 1996;67:1448–1456. [PubMed: 8858926]
- MacCumber MW, Ross CA, Snyder SH. Endothelin in brain: receptors, mitogenesis and biosynthesis in glial cells. *Proc Natl Acad Sci USA* 1990;87:2359–2363. [PubMed: 2156267]
- Marais R, Light Y, Mason C, Paterson H, Olson MF, Marshall CJ. Requirement of Ras-GTP-Raf complexes for activation of Raf-1 by protein kinase C. *Science* 1998;280:109–112. [PubMed: 9525855]
- Ming XF, Burgering BM, Wennstrom S, Claesson-Welsh L, Heldin CH, Bos JL, Kozma SC, Thomas G. Activation of p70/p85 S6 kinase by a pathway independent of p21ras. *Nature* 1994;371:426–429. [PubMed: 8090223]
- Nedergaard M. Direct signaling from astrocytes to neurons in cultures of mammalian brain cells. *Science* 1994;263:1768–1771. [PubMed: 8134839]

- Nishizuka Y. The role of protein kinase C in cell surface signal transduction and tumor promotion. *Nature* 1984;308:693–698. [PubMed: 6232463]
- Okumura N, Takimoto K, Okada M, Nakagawa H. C6 glioma cells produce basic fibroblast growth factor that can stimulate their own proliferation. *J Biochem (Tokyo)* 1989;106:904–909. [PubMed: 2613696]
- Pace AM, Faure M, Bourne HR. Gi2-mediated activation of the MAP kinase cascade. *Mol Biol Cell* 1995;6:1685–1695. [PubMed: 8590798]
- Parpura V, Basarsky TA, Liu F, Jeftinija S, Haydon PG. Glutamate-mediated astrocyte–neuron signalling. *Nature* 1994;369:744–747. [PubMed: 7911978]
- Reggiani A, Carenzi A, Della Bella D. Influence of opioids on β -receptors down-regulation: studies in cultured glioma C6 cells. *Brain Res* 1987;423:254–260. [PubMed: 2823987]
- Ruzicka BB, Fox CA, Thompson RC, Meng F, Watson SJ, Akil H. Primary astroglial cultures derived from several rat brain regions differentially express mu, delta and kappa opioid receptor mRNA. *Mol Brain Res* 1995;34:209–220. [PubMed: 8750824]
- Smart D, Smith G, Lambert DG. Mu-opioids activate phospholipase C in SH-SY5Y human neuroblastoma cells via calcium-channel opening. *Biochem J* 1995;305:577–582. [PubMed: 7832776]
- Smrcka AV, Sternweis PC. Regulation of purified subtypes of phosphatidylinositol-specific phospholipase C beta by G protein alpha and beta gamma subunits. *J Biol Chem* 1993;268:9667–9674. [PubMed: 8387502]
- Soltoff SP. Related adhesion focal tyrosine kinase and the epidermal growth factor receptor mediate the stimulation of mitogen-activated protein kinase by the G-protein-coupled P2Y2 receptor. Phorbol ester or $[Ca^{2+}]_i$ elevation can substitute for receptor activation. *J Biol Chem* 1998;273:23110–23117. [PubMed: 9722539]
- Soltoff SP, Avraham H, Avraham S, Cantley LC. Activation of P2Y2 receptors by UTP and ATP stimulates mitogen-activated kinase activity through a pathway that involves related adhesion focal tyrosine kinase and protein kinase C. *J Biol Chem* 1998;273:2653–2660. [PubMed: 9446569]
- Stehno-Bittel L, Krapivinsky G, Krapivinsky L, Perez-Terzic C, Clapham DE. The G protein beta gamma subunit transduces the muscarinic receptor signal for Ca^{2+} release in *Xenopus* oocytes. *J Biol Chem* 1995;270:30068–30074. [PubMed: 8530411]
- Stiene-Martin A, Hauser KF. Glial growth is regulated by agonists selective for multiple opioid receptor types in vitro. *J Neurosci Res* 1991;29:538–548. [PubMed: 1665190]
- Stiene-Martin A, Zhou R, Hauser KF. Regional, developmental, and cell cycle-dependent differences in mu, delta, and kappa-opioid receptor expression among cultured mouse astrocytes. *Glia* 1998;22:249–259. [PubMed: 9482211]
- Straus DS, Burke EJ. Glucose stimulates IGF-I gene expression in C6 glioma cells. *Endocrinology* 1995;136:365–368. [PubMed: 7828554]
- Thomas SM, DeMarco M, D'Arcangelo G, Halegoua S, Brugge JS. Ras is essential for nerve growth factor- and phorbol ester-induced tyrosine phosphorylation of MAP kinases. *Cell* 1992;68:1031–1040. [PubMed: 1312392]
- van Biesen T, Hawes BE, Luttrell DK, Krueger KM, Touhara K, Porfiri E, Sakaue M, Luttrell LM, Lefkowitz RJ. Receptor-tyrosine-kinase- and G beta gamma-mediated MAP kinase activation by a common signalling pathway. *Nature* 1995;376:781–784. [PubMed: 7651538]
- van Biesen T, Hawes BE, Raymond JR, Luttrell LM, Koch WJ, Lefkowitz RJ. G(o)-protein alpha-subunits activate mitogen-activated protein kinase via a novel protein kinase C-dependent mechanism. *J Biol Chem* 1996;271:1266–1269. [PubMed: 8576109]
- van Corven EJ, Hordijk PL, Medema RH, Bos JL, Moolenaar WH. Pertussis toxin-sensitive activation of p21ras by G protein-coupled receptor agonists in fibroblasts. *Proc Natl Acad Sci USA* 1993;90:1257–1261. [PubMed: 7679495]
- Verity AN, Wyatt TL, Hajos B, Eglen RM, Baecker PA, Johnson RM. Regulation of glial cell line-derived neurotrophic factor release from rat C6 glioblastoma cells. *J Neurochem* 1998;70:531–539. [PubMed: 9453547]

- Wood KW, Sarnecki C, Roberts TM, Blenis J. Ras mediates nerve growth factor receptor modulation of three signal-transducing protein kinases: MAP kinase, Raf-1, and RSK. *Cell* 1992;68:1041–1050. [PubMed: 1312393]
- Wu D, Katz A, Simon MI. Activation of phospholipase C beta 2 by the alpha and beta gamma subunits of trimeric GTP-binding protein. *Proc Natl Acad Sci USA* 1993;90:5297–5301. [PubMed: 8389480]

FIG. 1.

κ -Opioid stimulation of DNA synthesis in C6 cells. Cells were maintained in “low MEM” for 48 h before agonist and inhibitor treatment. Inhibitors were added to the same medium before opioid. [^3H]Thymidine ($0.5 \mu\text{Ci/ml}$) was included 30 min after U69,593 (U69) for the final 23 h in the presence of the other reagents. **A:** U69 stimulates [^3H]thymidine incorporation in a dose-dependent manner. Nonlinear regression analysis of the average $\pm$ SEM (bars) values of three independent experiments performed in triplicate indicates an EC_{50} of 1.03 nM. **B:** U69 ($1 \mu\text{M}$) stimulation of DNA synthesis is blocked by the following agents with the indicated concentrations and times before U69 treatment—the κ -OR antagonist nor-BNI ($1 \mu\text{M}$, 1 h), PTX (100 ng/ml, overnight), nifedipine ($1 \mu\text{M}$, 30 min), U73,122 ($1 \mu\text{M}$, 30 min), dantrolene ($1 \mu\text{M}$, 30 min), or BAPTA ($50 \mu\text{M}$, 30 min). *Significantly greater than all points, $p < 0.01$ by ANOVA. Data are mean $\pm$ SEM (bars) values from three to eight experiments performed in triplicate. Basal [^3H]thymidine incorporation was measured as $13,560 \pm 1,233$ dpm per well.

FIG. 2.

κ -Opioid stimulation of PI turnover. C6 cells were maintained in “low MEM” for 48 h and then labeled with $2 \mu\text{Ci/ml}$ *myo*-[^3H]inositol per well in the same medium for 24 h before activation. Medium was changed, and cells were incubated in “low MEM” plus 5 mM LiCl for 1 h before drug treatment. U69,593 (U69; $1 \mu\text{M}$) stimulation of PLC activity ensued for 1 h in the presence of the indicated inhibitors. The following reagents were applied at the indicated concentrations and times before U69 treatment: nor-BNI ($1 \mu\text{M}$, 1 h), PTX (100 ng/ml, overnight), nifedipine ($1 \mu\text{M}$, 30 min), and U73,122 ($1 \mu\text{M}$, 30 min). *Significantly greater than control, #significantly less than U69 alone, $p < 0.01$ by ANOVA. Data are mean $\pm$ SEM (bars) values from three to seven experiments performed in triplicate. Basal $^3\text{H-IP}_x$ levels were measured as $31,850 \pm 4,239$ dpm per well.

FIG. 3.

Inhibitors of PKC and MEK block κ -opioid stimulation of [³H]thymidine incorporation. C6 cells were maintained in “low MEM” for 48 h before treatment. Opioids, inhibitors, and [³H] thymidine were added to the same medium as described in Fig. 1. The following inhibitors were used at the concentrations and times before U69,593 (U69) treatment indicated: PMA (1 μ M, overnight), GFX (100 nM, 30 min), and PD98059 (1 μ M, 30 min). *Significantly greater than control, $p < 0.01$ by ANOVA. Data are mean $\pm$ SEM (bars) values from three to eight experiments performed in triplicate.

FIG. 4.

Time- and concentration-dependent κ -opioid phosphorylation of ERK1/2. C6 cells were maintained in “low MEM” for 48 h before treatment. Opioids were added to the same medium. Cell lysates were prepared, and 15 μ g of protein was loaded per lane on 10% sodium dodecyl sulfate-polyacrylamide mini-gels for western blot analysis. **A:** Time course. ERK1/2 phosphorylation was measured after exposure for 0.5, 1, 5, 10, 30, and 60 min to 5 nM U69,593. Data are mean $\pm$ SEM (bars) values of three to five separate experiments. **B:** Concentration dependence. ERK1/2 phosphorylation was assayed after a 5-min exposure to 0.5, 1, 5, 10, or 100 nM concentrations of U69,593. Data are mean $\pm$ SEM (bars) values of three to five separate experiments. Data were compiled by densitometric analysis (NIH Image software) and represented as the fold increased intensity as normalized by untreated controls in each experiment. Also shown are representative membranes blotted first with anti-phospho(P) ERK1/2 (**top**) and then stripped and reblotted with anti-ERK1 (**bottom**).

FIG. 5.

Inhibition of κ -opioid phosphorylation of ERK1/2. C6 cells were kept in “low MEM” for 48 h before treatment. Opioids and inhibitors were added to the same medium. Cell lysates were prepared, and 15 μ g of protein was loaded per lane on 10% sodium dodecyl sulfate-polyacrylamide mini-gels. U69,593 (U69; 10 nM, 10 min) was used to stimulate ERK1/2 phosphorylation. The following inhibitors were applied at the following concentrations and times before U69 treatment: (A) Nor-BNI (1 μ M, 1 h), PTX (100 ng/ml, overnight), nifedipine (1 μ M, 30 min), U73,122 (1 μ M, 30 min), dantrolene (1 μ M, 30 min), and BAPTA (50 μ M, 30 min) and (B) PMA (1 μ M, overnight), GFX (100 nM, 30 min), and PD98059 (1 μ M, 30 min). *Significantly greater than all points, $p < 0.01$ by ANOVA. Data are mean $\pm$ SEM (bars) values from three to nine experiments. Data were compiled by densitometric analysis (NIH Image software) and represented as the fold increased intensity as normalized by untreated controls in each experiment. Also shown are representative membranes blotted first with anti-phospho (P)ERK1/2 (top) and then stripped and reblotted with anti-ERK1 (bottom).

FIG. 6.

κ -OR mediates phosphorylation of ERK1/2 via $G\beta\gamma$ proteins. C6 cells were grown as described and then were transfected with 1 μ g of CD8 or CD8- β ARK-C cDNA and 2 μ l of LIPOFECTAMINE per well. Before agonist stimulation, cells were maintained in “low MEM” for 48 h. U69,593 (U69; 10 nM, 10 min) phosphorylation was expressed as fold activation over the respective transfected control (control = 1.0). CD8 and CD8- β ARK transfection was confirmed in parallel samples by immunoblotting with anti-CD8 Ab. *Significantly greater than un-stimulated CD8 vector controls, $p < 0.01$ by Student's t test; #significantly less than U69 plus CD8 vector, no different than CD8- β ARK control, $p < 0.001$ by Student's t test. Data are mean $\pm$ SEM (bars) values from three to five experiments. Data were compiled by

densitometric analysis (NIH Image software). Also shown is a representative membrane blotted first with anti-phospho(P)ERK1/2 (**top**) and then stripped and reblotted with anti-ERK1 (**bottom**).

FIG. 7.

N17Ras blocks κ -opioid phosphorylation of ERK1/2. Cells were grown as described and then were transfected with 1 μ g of N17Ras-pcDNAIII or the vector alone and 2 μ l of LIPOFECTAMINE per well. Before agonist stimulation, cells were maintained in “low MEM” for 48 h. U69,593 (U69; 10 nM, 10 min) phosphorylation was expressed as fold activation over the respective transfected control (control = 1.0). N17Ras transfection was confirmed (compared with vector-transfected cells) in parallel samples by immunoblotting with anti-Ras. *Significantly greater than un-stimulated vector controls, $p < 0.01$ by Student's t test; #significantly less than U69 plus vector, no different than N17Ras control, $p < 0.001$ by Student's t test. Data are mean $\pm$ SEM (bars) values from three to seven experiments. Data

were compiled by densitometric analysis (NIH Image software). Also shown are representative membranes blotted first with anti-phospho(P)ERK1/2 (**top**) and then stripped and reblotted with anti-ERK1 (**bottom**).

FIG. 8. κ -Opioid-stimulated PYK2 phosphorylation. Cells were plated equally in 100-mm-diameter dishes as described. After 48 h in “low MEM” cells were either treated with 10 nM U69,593 (U69) for 3 min or lysed immediately (untreated controls). After incubation with anti-phosphotyrosine Abs plus protein A-Sepharose for 4 h at 4°C, immunoprecipitates were resolved on 10% sodium dodecyl sulfate-polyacrylamide gels. Immunoblotting with anti-PYK2 Ab reveals increased phosphorylated PYK2 in treated cells. Shown are a representative blot and the average densitometry gained from three separate experiments. Data are average $\pm$ SEM (bars) densitometry from three experiments as normalized by the average of the control band densities. *Significantly greater than control, $p < 0.01$ by Student's t test.