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Kappa opioids promote the proliferation of astrocytes via Gβγ and β-arrestin 2-dependent MAPK-mediated pathways

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Abstract

GTP binding regulatory protein (G protein)-coupled receptors can activate MAPK pathways via G protein-dependent and -independent mechanisms. However, the physiological outcomes correlated with the cellular signaling events are not as well characterized. In this study, we examine the involvement of G protein and β-arrestin 2 pathways in kappa opioid receptor-induced, extracellular signal-regulated kinase 1/2 (ERK1/2)-mediated proliferation of both immortalized and primary astrocyte cultures. As different agonists induce different cellular signaling pathways, we tested the prototypic kappa agonist, U69593 as well as the structurally distinct, non-nitrogenous agonist, C(2)-methoxymethyl salvinorin B (MOM-Sal-B). In immortalized astrocytes, U69593, activated ERK1/2 by a rapid (min) initial stimulation that was sustained over 2 h and increased proliferation. Sequestration of activated Gβγ subunits attenuated U69593 stimulation of ERK1/2 and suppressed proliferation in these cells. Furthermore, small interfering RNA silencing of β-arrestin 2 diminished sustained ERK activation induced by U69593. In contrast, MOM-Sal-B induced only the early phase of ERK1/2 phosphorylation and did not affect proliferation of immortalized astrocytes. In primary astrocytes, U69593 produced the same effects as seen in immortalized astrocytes. MOM-Sal-B elicited sustained ERK1/2 activation which was correlated with increased primary astrocyte proliferation. Proliferative actions of both agonists were abolished by either inhibition of ERK1/2, Gβγ subunits or β-arrestin 2, suggesting that both G protein-dependent and -independent ERK pathways are required for this outcome.

Keywords: β-arrestins, astrocytes, extracellular signal-regulated kinase/mitogen-activated protein kinase, GTP binding regulatory proteins, opioid receptors, opioids.

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The binding of an agonist to a cell surface receptor has the potential to initiate numerous signaling cascades. In turn, diverse agonists are capable of initiating distinct pathways, and because of this functional selectivity such paths may be preferentially activated in certain cell types (for a review see Urban *et al.* 2007). A striking example of the multiplicity of signaling pathways is the recent finding that GTP binding regulatory protein (G protein)-coupled receptors (GPCRs) activate the extracellular signal-regulated kinase 1/2–mitogen-activated protein kinase (ERK1/2–MAPK) phosphorylation cascade by G protein-dependent and β-arrestin-dependent mechanisms in the same cell (Ahn *et al.* 2004; Barnes *et al.* 2005; Lefkowitz and Shenoy 2005; Gesty-Palmer *et al.* 2006;

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Abbreviations used: β-arr 2-GFP, β-arrestin 2 tagged with green fluorescent protein; Ab, antibody; BrdU, 5′-bromo-2′-deoxy-uridine; CD8-βARK-C, CD8-β-adrenergic receptor kinase; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; ERK1/2, extracellular signal-regulated kinase; FBS, fetal bovine serum; G protein, GTP binding regulatory protein; GFAP, glial fibrillary acidic protein; GPCR, G protein-coupled receptor; HA, hemagglutinin; HEK cells, human embryonic kidney cells; JNK, c-Jun N-terminal protein; KOR, κ opioid receptor; MAPK, mitogen-activated protein kinase; MOM-Sal-B, C(2)-methoxymethyl salvinorin B; norBNI, norbinaltorphimine; PBS, phosphate-buffered saline; PTX, pertussis toxin; RT, room temperature; siRNA, small interfering RNA; TBST, Tris-buffered saline + 0.2% Tween 20.

Shenoy *et al.* 2006). In many of these studies, agonist-induced ERK1/2 phosphorylation in receptor-transfected human embryonic kidney (HEK293) cells was determined to be mediated via a G protein-coupled mechanism involving protein kinase A and/or protein kinase C during the early phase of activation. The phospho-ERK generated in this paradigm is then translocated to the nucleus to activate transcriptional substrates. In contrast, sustained ERK1/2 activation entails a G protein-, protein kinase A-, and protein kinase C-independent pathway in which β-arrestins play a pivotal role (Tohgo *et al.* 2002). The phospho-ERK produced by this mechanism is retained in the cytoplasm. Although these two independent ERK1/2 signaling pathways can have different effects on transcription, their impacts on outcomes of physiological regulation are not well characterized (Barnes *et al.* 2005; Lefkowitz *et al.* 2006).

The emerging status of astrocytes as equal partners in synaptic transmission has been supported by the recent discovery of a number of 'gliotransmitters' (Diamond 2006; Seifert *et al.* 2006). These include D-serine, glutamate, leukemia inhibitory factor, ephrins, and thrombospondins that are secreted by astrocytes and play dynamic roles in synaptic signaling, myelination and neurogenesis (Yang *et al.* 2003; Christopherson *et al.* 2005; Ishibashi *et al.* 2006; Panatier *et al.* 2006; Nishida and Okabe 2007; Jiao *et al.* 2008). As astrocytes are a functionally and morphologically diverse group of cells, it is important to understand how cell division of each type is regulated to facilitate communication with neurons (Raff *et al.* 1983; Wang *et al.* 1994; Muroyama *et al.* 2005). MAPK phosphorylation cascades have been implicated in proliferation, differentiation, and survival of primary astrocytes induced by various mitogens (Biesiada *et al.* 1996; Kurino *et al.* 1996; Lazarini *et al.* 1996; Riboni *et al.* 2000, 2001; Fanton *et al.* 2001; Lenz *et al.* 2001). The opioid GPCRs have been demonstrated to regulate ERK1/2/MAPKs in immortalized and primary astrocytes, in some pathways by transactivation of growth factor receptors (Belcheva *et al.* 2003, 2005; Mahajan *et al.* 2005; Bruchas *et al.* 2006). Opioid receptor regulation of glial cell proliferation during CNS development and injury has also been well documented *in vivo* and *in vitro* (Hauser and Stiene-Martin 1991; Stiene-Martin *et al.* 1991; Barg *et al.* 1993, 1994; Hauser and Mangoura 1998; Xu *et al.* 2007).

The current study focuses on temporal and mechanistic aspects of κ-opioid receptor (KOR) activation of ERK1/2 and how this affects growth of astrocytes. In this study, we measure ERK1/2 activation by distinct κ-opioid agonists, C(2)-methoxymethyl salvinorin B (MOM-Sal-B) and U69593 in rat cortical immortalized type-1 and primary (type-1 and type-2) astrocytes. We find that the two ligands promote different temporal signaling patterns with respect to ERK activation. This functional selectivity is dependent on the cellular environment as KOR ligand differences are only detected in immortalized cells. Furthermore, we find that KOR-mediated primary astrocyte proliferation involves both Gβγ- and β-arrestin 2-mediated ERK1/2 activation.

Experimental procedures

Reagents

Chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA) with the following exceptions: U69593 and norbinaltorphimine (norBNI) were from NIDA Drug Supply (Research Triangle, NC, USA); epidermal growth factor (EGF) and U0126 from Calbiochem (San Diego, CA, USA); trypsin–EDTA solution from Gibco (Carlsbad, CA, USA); pertussis toxin (PTX) from List Biological Laboratories, Inc. (Campbell, CA, USA); Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) from ATCC (Manassas, VA, USA); salvinorin A was isolated from *Salvia divinorum* and purified to > 98% homogeneity; the salvinorin A derivative, MOM-Sal-B was a generous gift from Dr David Y. W. Lee, McLean Hospital (Belmont, MA, USA). Anti-phospho-ERK1/2 (directed against phospho-Thr202/Tyr204) antibody (Ab) was from Cell Signaling Technology (Beverly, MA, USA); anti-glial fibrillary acidic protein (GFAP) Ab was from ImmunoStar, Inc. (Hudson, WI, USA; Catalog #22522); anti-β-arrestin 2 and anti-ERK Ab were from Santa Cruz (Santa Cruz, CA, USA); anti-TuJ 1 Ab was from Neuromics (Edina, MN, USA); 5′-bromo-2′-deoxy-uridine (BrdU) and the Abs for its detection from the BrdU Labeling and Detection Kit I (Roche, Basel, Switzerland), Alexa Fluor labeled secondary Abs and horse serum from Invitrogen–Molecular Probes (Carlsbad, CA, USA); and VECTASHIELD Mounting Medium (Vector Laboratories, Inc., Burlingame, CA, USA). CD8 and the Gβγ scavenger, CD8-β-adrenergic receptor kinase (CD8-βARK-C) cDNA, were a generous gift from S. Gutkind (NIH, Bethesda, MD, USA). Small interfering RNA (siRNA) directed against the β-arrestin 2 gene and non-targeting control siRNA were purchased from Dharmacon RNA technologies (Lafayette, CO, USA).

Primary astrocyte cultures

Postnatal day 1 Sprague–Dawley rat pups were killed and their cortical regions were dissected out, minced, suspended in 2.5 g/mL ice-cold phosphate-buffered saline (PBS), and trypsinized by incubation with an equal volume of 0.05% trypsin–EDTA solution at 37°C for 15 min. The tissue was pelleted (1000 $g \times 10$ min), resuspended in 5 g/mL DMEM containing 5% FBS and 5% horse serum, triturated, and plated onto poly-L-lysine (mol. wt. 30 000–70 000) coated tissue culture flasks as indicated.

After 7 days in culture, the poly-L-lysine coated flasks were shaken for at least 2 h, after which the unattached cells were removed and fresh culture medium was added (DMEM, 5% FBS, and 5% horse serum EGF). For ERK1/2 assay, growth medium was replaced with DMEM without serum 24 h prior to ligand treatment. Of the total number of cells in primary cultures, 90% were GFAP positive and < 1% were TuJ 1 (neuronal marker) positive. Generally, primary rat cortical astrocyte cultures were morphologically heterogeneous in that they contained both GFAP-positive type-1 astrocytes that are flat polyhedral-shaped cells and type-2 that are spindle shaped and possess two or more processes. Primary cultures from some tissues such as neonatal mouse spinal cord only contain type-2 astrocytes whereas primary neonatal mouse brain astrocyte cultures consist of predominantly type-1 cells (Raff *et al.* 1983;

Hauser and Stiene-Martin 1991; Xu *et al.* 2007). Animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

Type-1 immortalized rat cortical astrocyte cultures

Rat cortical astrocytes (CTX TNA2; ATCC), were established from cultures of primary type-1 astrocytes from 1-day-old rat brain frontal cortex and grown as described (Radany *et al.* 1992; Belcheva *et al.* 2003, 2005).

Transient transfections

Immortalized rat astrocytes were transiently transfected with pcDNA3 (for mock transfections) or KOR cDNA (pCMV-neo expression vector) using FuGENE 6 (Roche, Indianapolis, IN, USA) transfection reagent following the manufacturer's instructions and adding 1 μg of cDNA and 3 μL of transfection reagent. In some cases, cells were co-transfected with 1 μg CD8 (in pcDNAI AMP vector) or CD8-βARK-C in pcDNAIII vector cDNA using FuGENE 6 as described (Belcheva *et al.* 1998). CD8-βARK-C expresses a membrane anchoring protein (CD8) fused to the βγ subunit-binding segment of the carboxyl terminus of βARK (Crespo *et al.* 1995).

Small interfering RNA preparation and transfection

Small interfering RNA targeting the rat β-arrestin 2 gene was designed and synthesized by Dharmacon RNA technologies. The following siRNA preparations were used: siGENOME standard SMART pool to rat ARRB2 (Catalog #D-080157-00, target sequences: GGAGCUACCUUUCGUCCUA, GAUGAAGGAUGACGACUGU, GAGAAGACCUGGAUGUACU, and GCAAAGAUCUGUUCAUCGC) and siCONTROL non-targeting siRNA pool (negative control that has been bioinformatically designed and validated to not have any known targets, Catalog #D-001206-13, target sequences: AUGAACGUGAAUUGCUCAA, UAAGGCUAUGAAGAGAUAC, AUGUAUUGGCCUGUAUUAG, and UAGCGACUAAACACAUCAA). The siRNA preparations were resuspended in Dharmacon-provided siRNA buffer to a stock concentration of 20 μM. Immortalized and primary rat astrocytes were transiently transfected using the Amaxa Nucleofector electroporator (Amaxa Biosystems, Gaithersburg, MD, USA). Briefly, cells were removed from flasks by treatment with 0.05% trypsin and 0.02% EDTA for 1 min at 37°C, washed with media and incubated for 2 h at 37°C in a 50 g/mL conical tube. Equal amount of cells, no more than 2.0×10^6, were distributed in tubes, harvested by centrifugation (1000 *g*) and resuspended in 100 μL rat astrocyte nucleofector solution (Amaxa Biosystems). Two micrograms of KOR cDNA and 1 μM control or target siRNAs were added to each tube with cells and electroporation was performed using optimal Amaxa Nucleofector program T-20. This program was developed by Amaxa specifically for primary astrocytes. The transfection efficiency of Amaxa electroporation is much higher than the 9 ± 1% that we obtained with FuGENE 6 as determined by Gal expression measurements. The Amaxa estimate for rat astrocyte transfection is 70% using their T-20 program. To document this, cells were transfected with enhanced green fluorescent protein, along with GFAP and DAPI and their staining were compared. The majority of $GFAP^+$ cells were labeled with enhanced green fluorescent protein with varying degrees of staining. Immediately following electroporation, cells were transferred to six-well tissue culture plates or eight-well chambers containing growth media and cultured overnight at 37°C. Then cells were washed three times with media deprived of serum and cultured for an additional 24 h in fresh growth media. Cells were grown for 24 h in serum-deprived media prior to initiation of ERK1/2 activity or proliferation determinations. The efficiency of gene silencing was validated by immunoblotting with corresponding Abs.

Extracellular signal-regulated kinase 1/2 assays

Extracellular signal-regulated kinase 1/2 phosphorylation was measured by immunoblotting as described (Belcheva *et al.* 2001). Cells were treated first with either inhibitors or antagonist, and then with MOM-Sal-B or U69593 as described in the Figure legends. Cells were then washed with PBS and lysed with buffer containing 20 mM HEPES, 10 mM EGTA, 40 mM β-glycerophosphate, 2.5 mM $MgCl_2$, 2 mM sodium vanadate, 1% Nonidet-40, 1 mM phenylmethylsulfonyl fluoride, 20 μg/mL aprotinin, and 20 μg/mL leupeptin. Cell lysates were centrifuged at 14 000 *g* for 20 min at 4°C and protein concentration of the supernatants was determined. Samples (10–20 μg protein/lane) were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins were blotted on Immobilon P™ PVDF membranes (Millipore, Bedford, MA, USA). Non-specific sites were blocked with 5% milk in Tris-buffered saline + 0.2% Tween 20 (TBST). Blots were then washed three times with TBST and incubated with anti-phospho-ERK1/2 Ab for at least 15 h at 4°C. After three washes with TBST, blots were incubated with 1 : 2000 diluted horseradish peroxidase-conjugated goat anti-mouse-IgG for 1 h at 25°C. For assurance of equivalent total ERK1/2 protein per lane, representative blots were stripped (0.2 M glycine, pH 2.5, 60 min at 25°C) and exposed to anti-ERK1/2 Ab (1 : 1000), followed by 1 : 20 000 diluted horseradish peroxidase-conjugated goat anti-rabbit-IgG. Bands were visualized using an ECL chemiluminescence detection system (GE Healthcare, Buckinghamshire, UK). Band intensities were determined by densitometry using photos taken with a Kodak DC120 digital camera, Kodak ds 1D version 3.0.2 (Scientific Imaging Systems, Kodak Digital Science, New Haven, CT, USA) and analyzing them with NIH IMAGEJ version 1.32 software. ERK stimulation in opioid-treated cells is expressed as fold change over basal levels in untreated cells or percentage maximal expression.

Confocal imaging of HA-KOR and β-arrestin 2-GFP transfected HEK293 cells

Human embryonic kidney 293 cells were cultured in minimum essential medium (Invitrogen) containing 10% heat-inactivated FBS (Invitrogen) and 1% penicillin–streptomycin (Invitrogen) at 37°C under 5% CO_2. The HEK293 cells were transiently transfected with hemagglutinin (HA-N-terminus) tagged rat KOR (5 μg cDNA) and β-arrestin 2 tagged with green fluorescent protein (β-arr 2-GFP) (2 μg cDNA). Live cell Ab staining of transfected HA-KOR was obtained by incubating cells in serum-free minimum essential medium for 30 min with anti-HA-Alexa Fluor 594 conjugate (1 : 100; Molecular Probes/Invitrogen). Agonists were added and multiple cells were imaged per dish. HEK293 cell transfection by electroporation and confocal imaging were performed as previously described (Bohn *et al.* 2004).

Immunocytochemical detection of BrdU incorporation and GFAP co-staining

Cells were grown in poly-L-lysine coated eight-well chamber slides until they were about 50% confluent in the case of primary or about 60% confluent for immortalized astrocytes and growth medium was replaced with DMEM without serum for 28 h. They were then treated with opioids and/or inhibitors for 24 h and were labeled with 10 μM BrdU for the final 4 h of treatment. Cells were fixed with 4% *p*-formaldehyde in PBS for 20 min at 25°C. After 3 × 5 min PBS washes, cells were incubated with 2 N HCl/0.5% Triton X-100 in 0.1 × PBS for 1 h at 25°C, washed once for 5 min with PBS, pH 8.4, and 3 × 5 min with regular PBS, and blocked with PBS containing 0.5% bovine serum albumin and 0.1% Tween 20 for 30 min. BrdU and the Abs for its detection were used following the manufacturer's directions. Cells were incubated with rabbit anti-GFAP Ab (1 : 1 dilution) in blocking solution overnight at 4°C and washed 3 × 5 min with PBS. Then, cells were treated with mouse anti-BrdU monoclonal Ab, 1 : 10 dilution for 30 min at 37°C, washed 3 × 5 min with PBS and then incubated with both secondary Abs together in blocking solution (see above) for 1 h at 25°C (red fluorescence emitting Alexa Fluor 594 goat anti-rabbit IgG, highly cross-adsorbed Ab, 2 mg/mL, diluted 1 : 700 (for GFAP detection) and the green fluorescence emitting fluorescein-conjugated anti-mouse IgG Ab (1 : 10 dilution) from the kit for BrdU detection. After three PBS washes, slides were mounted using anti-fade VECTASHIELD mounting medium and covered with coverslips. Slides were examined for immunofluorescence with an Olympus AH-3 microscope (Tokyo, Japan) attached to a Soft Imaging Systems F-view II CCD digital camera (Tokyo, Japan) that has simultaneous recording capability of dual fluorescence label images.

Protein assay and statistical analysis

Protein concentrations were determined by the Bradford method (Bradford 1976) with bovine serum albumin (1 mg/mL) as standard. In all experiments reported, each *n* value represents a result gained from one culture. In primary culture studies, cells from at least two different litters of rat pup brains were used for each final determination. Statistical determinations were made by Student's *t*-

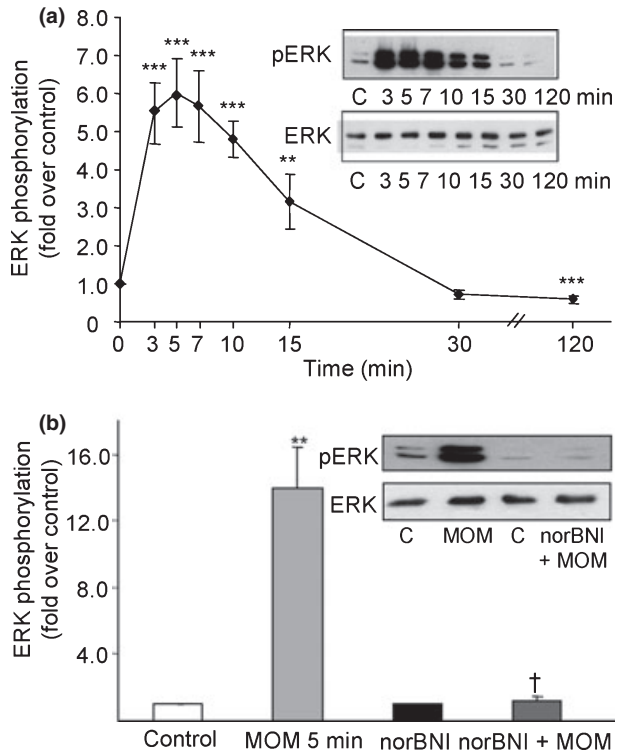


Fig. 1 Time course of MOM-Sal-B stimulation of ERK1/2 phosphorylation and antagonism by the selective KOR antagonist, norBNI in type-1 immortalized astrocytes transiently transfected with KOR cDNA. (a) Cells were treated with 1 μM MOM-Sal-B and assayed at the indicated time intervals; $n = 4–8$, $^{**}p < 0.01$ and $^{***}p < 0.001$ versus control. (b) Cells were pre-treated with 1 μM norBNI for 1 h, followed by a 5 min treatment with 1 μM MOM-Sal-B; $n = 4$, $^{**}p < 0.01$ versus control and $^{\dagger}p < 0.05$ versus agonist alone. The gels are representative immunoblots showing phosphorylated ERK1/2 and total ERK1/2 bands. The graph and curves show quantified ERK1/2 phosphorylation based on phospho-ERK1/2 to ERK ratios.

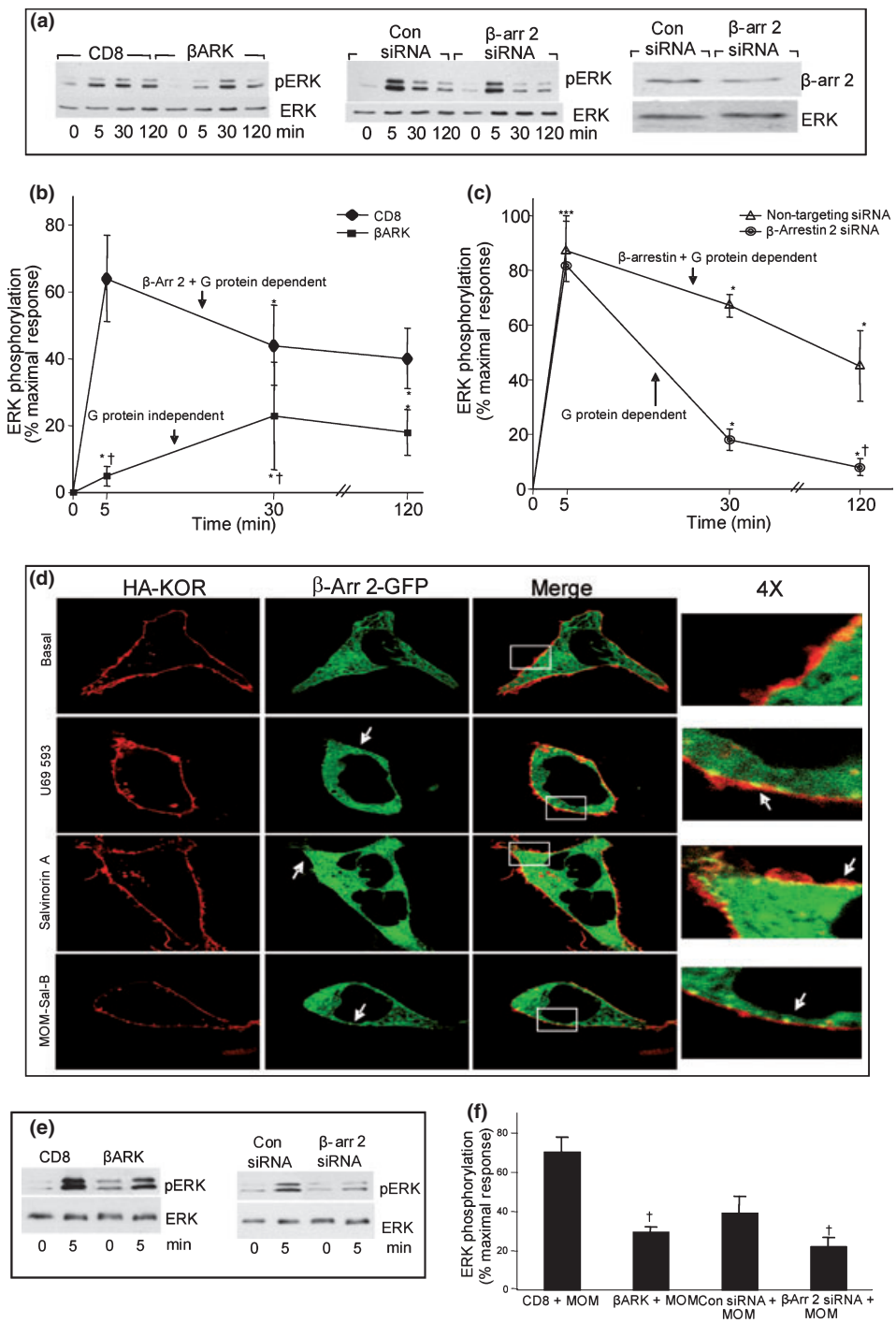
Fig. 2 Selective inhibition of early and later phases of U69593- and MOM-Sal-B-induced stimulation of ERK1/2 phosphorylation in type-1 immortalized astrocytes. (a) Effect of CD8-βARK-C and β-arrestin 2 siRNA on U69593 signaling. Cells were transiently transfected with KOR ± CD8 or CD8-βARK-C cDNA; or KOR cDNA ± non-targeting control or β-arrestin 2 siRNA. After 24 h growth in serum-deprived media, cells were treated with 1 μM U69593 for the indicated time intervals and ERK1/2 phosphorylation was assayed. The gels (left and center) are representative immunoblots showing phosphorylated ERK1/2. The gel on the right is a representative immunoblot showing β-arrestin 2 levels in cells transfected with non-targeting control or β-arrestin 2 siRNAs. (b and c) Figures show curves of quantified percentage maximal response of ERK1/2 phosphorylation for each treatment in the experiment outlined in panel (a); $n = 6–8$, $^{*}p < 0.05$ and $^{***}p < 0.001$ versus zero time control; $^{\dagger}p < 0.05$ versus their respective control treatment (CD8 or non-targeting siRNA) at the same time point. (d) Agonist-induced β-arrestin 2 translocation to KOR in living HEK293 cells. HEK293 cells were transiently transfected with HA-KOR and β-arr 2-GFP. HA-KOR were labeled with anti-HA antibody conjugated to Alexa Fluor 594. Cells were treated with MOM-Sal-B (100 nM), salvinorin A (100 nM, provided by Thomas E. Prisinzano) or U69593 (100 nM) and images were taken at 5 min post-treatment. Representative cells are shown. β-Arr 2-GFP puncta at cell surface are indicated by white arrows. (e and f) Effect of CD8-βARK-C and β-arrestin 2 siRNA on MOM-Sal-B signaling. Cells were transiently transfected with KOR ± CD8 or CD8-βARK-C cDNA; or KOR cDNA ± non-targeting control or β-arrestin 2 siRNA, starved for 24–28 h, treated with 1 μM MOM-Sal-B for 5 min and ERK1/2 phosphorylation was assayed. The gels are representative immunoblots showing phosphorylated ERK1/2 and total ERK1/2 bands. The graph shows quantified % maximal response of ERK1/2 phosphorylation based on phospho-ERK1/2 to ERK ratios. $n = 3$; $^{\dagger}p < 0.05$ versus MOM-Sal-B alone.

test analysis using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). Data are expressed as the mean ± SEM.

Results

KOR activation of ERK1/2 in immortalized astrocytes

A time course study of the stimulation of ERK1/2 phosphorylation by salvinorin A was conducted with type-1 immortalized rat cortical astrocytes. This non-nitrogenous, highly selective κ-opioid agonist stimulated ERK phosphorylation 3.6-fold at the 5 min interval ($p < 0.05$ vs. control), but activation was not detected at 15 or 120 min. Salvinorin A is known to undergo a slow (~20–30 min) hydrolysis of its ester bond at C-2 *in vitro* and as short as 8 min in primates *in vivo* (Roth *et al.* 2002; Yan and Roth 2004; Hooker *et al.* 2008). MOM-Sal-B is a salvinorin A analog that has a more



stable methoxymethyl moiety at C(2), a high binding affinity for KOR, sevenfold greater potency in GTPγS binding assays and a longer half life *in vitro* than salvinorin A (Lee *et al.* 2005). Therefore, a time course study of the stimulation of ERK1/2 phosphorylation by MOM-Sal-B was conducted with type-1 immortalized rat cortical astrocytes (Fig. 1a). The κ opioid selective antagonist, norBNI abolished ERK1/2 activation by MOM-Sal-B implicating KOR in the signaling pathway (Fig. 1b). The MOM-Sal-B results were compared with U69593 data previously obtained for these same cells (Belcheva *et al.* 2003). While the κ opioid prototypic agonist, U69593, induced a sustained activation of ERK1/2 lasting over 2 h, MOM-Sal-B elicited a short-lived increase in ERK1/2 phosphorylation (< 30 min). As MOM-Sal-B also stimulated ERK1/2 phosphorylation robustly but transiently, the waning effects on the extent of ERK1/2 phosphorylation were not likely to be attributable to the degradation of the agonist (Fig. 1a).

Signaling mechanisms involved in κ opioid induced acute and sustained ERK1/2 phosphorylation

In immortalized astrocytes, U69593 elicits both rapid (min) and sustained (h) activation of ERK1/2, while MOM-Sal-B induces only the early phase suggesting that multiple KOR-mediated ligand specific, pathways of ERK1/2 activation may exist in astrocytes. It has been previously demonstrated that the early phase of ERK1/2 phosphorylation may be mediated by a G protein-dependent mechanism that is distinct from a later sustained activation which involves β-arrestins (Ahn *et al.* 2004; Gesty-Palmer *et al.* 2006). Therefore, we used a series of inhibitor and siRNA knockdown studies to assess the nature of the rapid and sustained phases of ERK1/2 activation in response to the two ligands. An earlier study by our group suggested that the rapid phase of ERK1/2 phosphorylation is G protein-dependent and may be mediated by the Gβγ subunit of heterotrimeric G proteins (Belcheva *et al.* 2005). The role of the Gβγ subunit was assessed here by over-expressing CD8-βARK-C cDNA, a chimeric molecule comprised of the CD8 receptor and the carboxyl terminus of the β-adrenergic receptor kinase that acts as a scavenger of Gβγ subunits (Crespo *et al.* 1995). As seen in Fig. 2a and b, CD8-βARK-C almost completely abolished the rapid phase and significantly attenuated the sustained phase of U69593-induced ERK1/2 phosphorylation (see curve labeled G protein independent in Fig. 2b). Transfection of the CD8 cDNA construct had no effect on KOR mediated ERK activation.

To test the role of β-arrestin 2 in the agonist-induced KOR activation of ERK in our astrocytic model, we utilized a siRNA silencing approach. Immortalized astrocytes were transfected with siRNAs directed against β-arrestin 2 or a non-targeting control by electroporation. Figure 2a (*right*-last two lanes) demonstrates that the β-arrestin 2 protein content was reduced by 40 ± 5%, $p < 0.05$, $n = 4$) by the siRNA in comparison to non-targeting control siRNA. U69593-induced ERK1/2 phosphorylation was reduced by the knockdown of β-arrestin 2; however, the temporal pattern differed greatly from that engendered by the Gβγ protein scavenger (Fig. 2a and c). While the late phase of U69593-induced ERK1/2 phosphorylation was significantly diminished, the early phase was not affected upon β-arrestin 2 silencing (see curve labeled 'G protein dependent' in Fig. 2c). These results suggest that U69593 activation of KOR results in an early phase of ERK1/2 activation that is mainly because of a Gβγ-mediated pathway accompanied by a late phase which is driven predominantly by β-arrestin 2.

To demonstrate an agonist-induced interaction between KOR and β-arrestin 2, we utilized a β-arr 2-GFP translocation assay in HEK293 cells expressing an HA-tagged KOR (Barak *et al.* 1997; Groer *et al.* 2007). In Fig. 2d, we

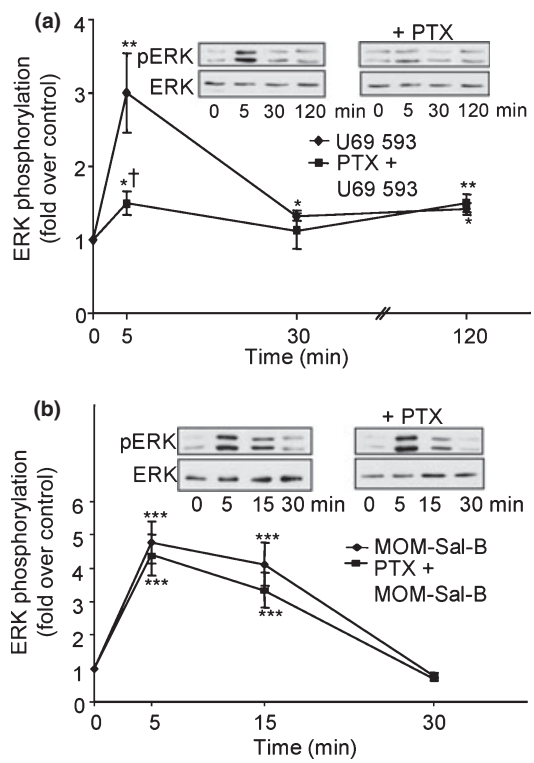


Fig. 3 Attenuation of κ opioid-induced stimulation of ERK1/2 phosphorylation by PTX in type-1 immortalized astrocytes. (a) Effect of PTX on U69593 signaling. Cells were transiently transfected with KOR cDNA, serum starved for 24 h in the presence of 100 ng/mL PTX and treated with 1 μM U69593 for the indicated time intervals and ERK1/2 phosphorylation was assayed; $n = 4$–10, $^{*}p < 0.05$ and $^{**}p < 0.01$ versus control; $^{\dagger}p < 0.05$ versus U69593 alone at the same time point. (b) Lack of effect of PTX on MOM-Sal-B signaling. Cells were transiently transfected with KOR cDNA, serum starved for 24 h in the presence of 100 ng/mL PTX and treated with 1 μM MOM-Sal-B for the indicated time intervals and ERK1/2 phosphorylation was assayed; $n = 4$–11, $^{***}p < 0.001$ versus control.

demonstrate that U69593, salvinorin A and MOM-Sal-B can induce β-arrestin 2 recruitment to the plasma membrane within 5 min of stimulation.

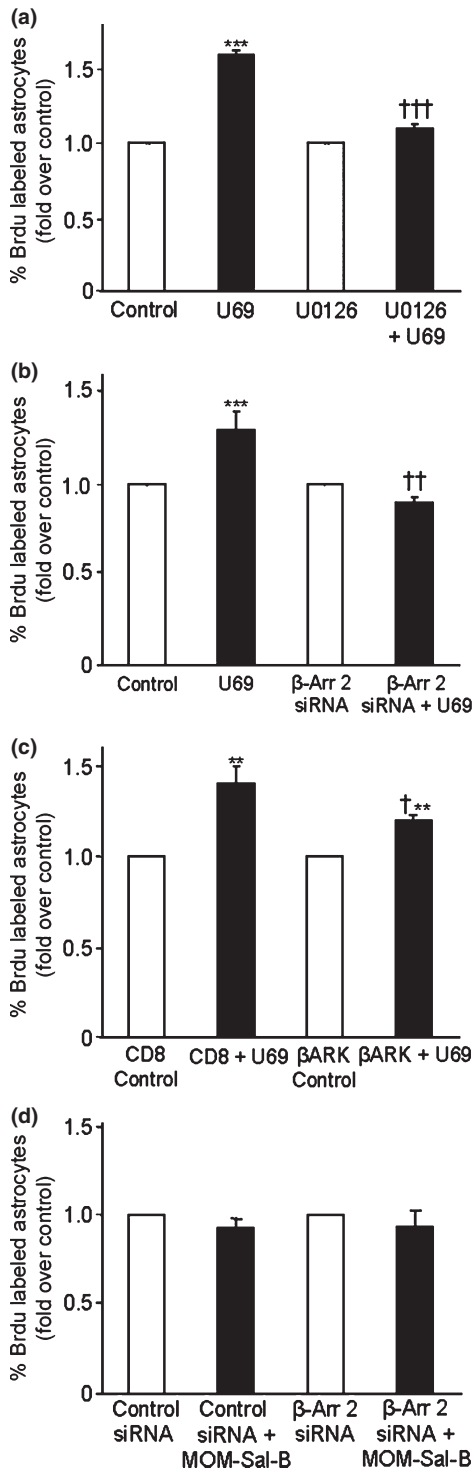
C(2)-methoxymethyl-Sal-B stimulated only the early phase of ERK1/2 activation in the immortalized astrocytes and accordingly, CD8-βARK-C diminished MOM-Sal-B (5 min) induced ERK1/2 phosphorylation by 59% in these cells (Fig. 2e) demonstrating the importance of the Gβγ subunit in this early signaling event. However, siRNA targeting β-arrestin-2 also attenuated the MOM-Sal-B activation of ERK at the early time point, suggesting that in response to this ligand, the early phase consists of additive G protein- and β-arrestin-2-mediated ERK phosphorylation (Fig. 2f).

To further establish that the early phase of ERK phosphorylation is transduced by a G protein-dependent mechanism, we treated the cells with the Gi/o inhibitor, PTX. Accordingly, PTX attenuated primarily the early phase of U69593-induced ERK phosphorylation (Fig. 3a). Surprisingly, PTX did not affect MOM-Sal-B stimulation of ERK phosphorylation (Fig. 3b). Therefore, it appears that only U69593 utilizes a PTX-sensitive Gi/o α protein. As the Gβγ scavenger diminished MOM-Sal-B stimulated ERK phosphorylation (Fig. 2f), the data suggest that MOM-Sal-B is acting via a PTX-insensitive G protein. There is precedent for opioid signaling to ERK via PTX-insensitive G proteins (Belcheva *et al.* 2000; Bruchas *et al.* 2007). In addition, PTX-insensitive mediation by Gβγ subunits of serotonin signaling to phospholipase D has been reported (McGrew *et al.* 2002).

Fig. 4 Inhibition of κ opioid-induced stimulation of type-1 immortalized astrocyte proliferation. (a) Effect of U0126 on U69593 signaling. Cells were transiently transfected with KOR cDNA, starved for 28 h and treated for the last 24 h with 1 μM U69593 and 1 μM U0126. BrdU was added for the last 20–30 min of treatment. In these studies > 2500 cells were counted for each determination; $n = 4$, $^{***}p < 0.001$ versus control and $^{\dagger\dagger\dagger}p < 0.001$ versus U69593 treated cells. (b) Effect of β-arrestin 2 siRNA on U69593 signaling. Cells were transiently transfected with KOR cDNA ± non-targeting or β-arrestin 2 siRNA. After 24 h on growth medium, they were starved for 28 h and treated for the last 24 h with 1 μM U69593. BrdU was added for the last 20–30 min of treatment. In these studies, ≥ 850 cells were counted for each determination; $n = 3$–5, $^{***}p < 0.001$ and $^{\dagger\dagger}p < 0.01$ versus control. (c) Effect of CD8-βARK-C on U69593 signaling. Cells were transiently transfected with KOR ± CD8 or CD8-βARK-C cDNA. After 24 h on growth medium, they were starved for 28 h and treated for the last 24 h with 1 μM U69593. BrdU was added for the last 20–30 min of treatment. In these studies, ≥ 850 cells were counted for each determination; $n = 3$, $^{**}p < 0.01$ versus control and $^{\dagger}p < 0.01$ versus their CD8 control in the presence of U69593. (d) Effect of β-arrestin 2 siRNA on MOM-Sal-B signaling. Cells were transiently transfected with KOR cDNA and control, non-targeting or β-arrestin 2 siRNA). After 24 h on growth medium, they were starved for 28 h and treated for the last 24 h with 1 μM MOM-Sal-B. BrdU was added for the last 20–30 min of treatment. In these studies, ≥ 3000 cells were counted for each determination; $n = 3$–4.

Effects of κ opioids on immortalized astrocyte proliferation as measured by BrdU counting

Having obtained evidence for two mechanisms of ERK1/2 activation, the question arises as to the physiological outcome of each. As opioid regulation of glial cell proliferation has been well established during CNS development



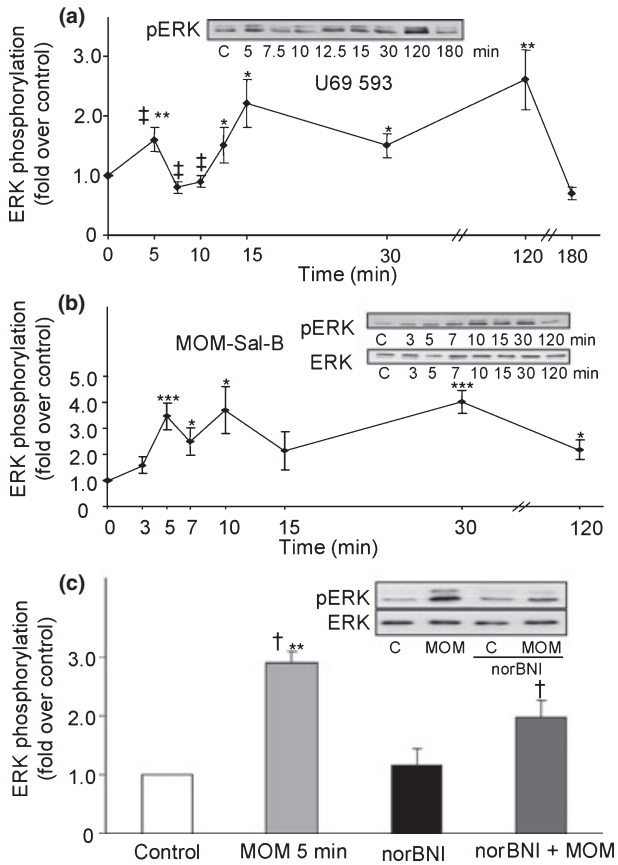


Fig. 5 Time course of MOM-Sal-B and U69593 modulation of ERK1/2 phosphorylation in type-1 and type-2 primary rat astrocytes. Cells were treated with 1 μM U69593 (a) or 1 μM MOM-Sal-B (b) for specific time intervals. U69593: $n = 7–12$. MOM-Sal-B: $n = 5–6$. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus control and $^{‡}p < 0.05$, the 5 min peak is significantly different from the 7.5 and 10 min points. (c) Cells were pre-treated with 1 μM norBNI for 1 h, followed by a 5 min treatment with 1 μM MOM-Sal-B; $n = 4$, $^{**}p < 0.01$ versus control and $^{†}p < 0.05$ versus agonist alone. The gels are representative immunoblots showing phosphorylated ERK1/2 and total ERK1/2 bands. The graphs and curves show quantified ERK1/2 phosphorylation based on phospho-ERK1/2 to ERK1/2 ratios.

and injury (Hauser and Stiene-Martin 1991; Stiene-Martin *et al.* 1991; Barg *et al.* 1993, 1994; Hauser and Mangoura 1998; Xu *et al.* 2007), we focused on the impact of the blockade of each of these pathways on astrocyte proliferation. When proliferation of type I immortalized astrocytes transfected with KOR was measured by BrdU counting after vehicle or U69593 treatment, the κ agonist elicited a 1.6-fold increase in proliferation that was abolished by U0126 indicating the dependency of ERK1/2 (Fig. 4a). U69593-induced proliferation was also blocked by β-arrestin 2 targeting siRNA (Fig. 4b). CD8-βARK-C over-expression resulted in a 52% decrease in U69593 stimulation of immortalized astrocyte proliferation (Fig. 4c). These results indicated that both ERK1/2-mediated pathways contribute to U69593-induced immortalized astrocyte proliferation.

Interestingly, MOM-Sal-B failed to stimulate proliferation of the immortalized astrocytes in the absence or presence of β-arrestin 2 siRNA suggesting that the late phase β-arrestin 2-dependent pathway may be required for KOR-induced proliferation in this cell line (Fig. 4d).

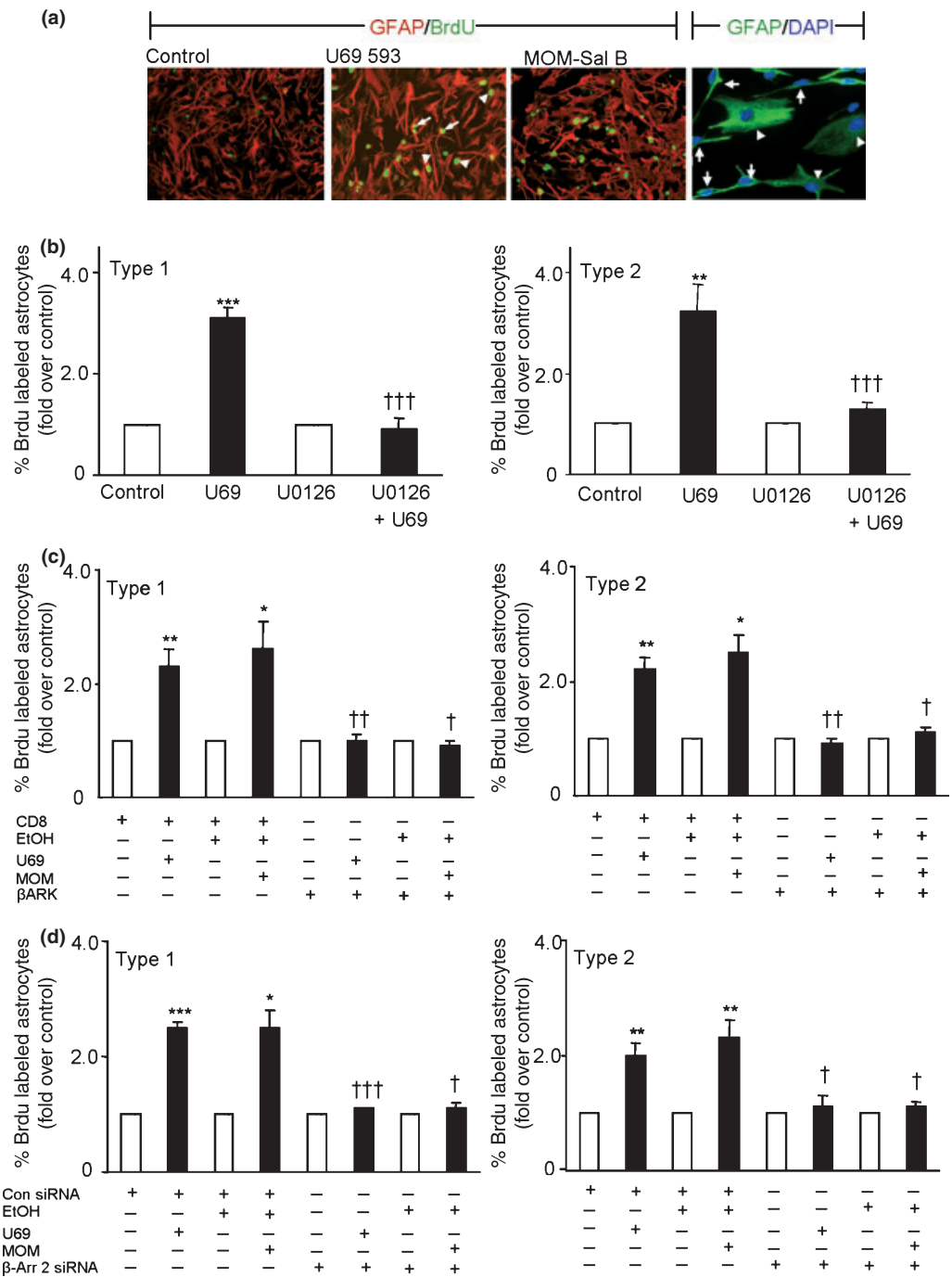
KOR stimulation of ERK and cell proliferation in primary rat astrocytes

As the cellular environment can impact the receptor signaling potential as much as the ligand, we tested whether the signaling and physiological effects imparted by the KOR were conserved in primary astrocyte cultures. The use of primary cultures of astrocytes, may serve as a more physiologically relevant model system in which to study the mechanism of KOR-mediated effects on cell growth.

In primary astrocytes, both U69593 and MOM-Sal-B, activated ERK1/2/MAPK via endogenous KOR by a rapid (min) initial stimulation that persisted up to 2 h (Fig. 5a and b). The long duration of MOM-Sal-B activation of ERK1/2 contrasted with its transient action in immortalized astrocytes (Fig. 1b). Cell type specific variations in the duration of GPCR ligand-induced ERK1/2 activation have been reported previously (Shah *et al.* 2003). The parallel

Fig. 6 Inhibition of κ opioid-induced stimulation of type-1 and type-2 primary astrocyte proliferation. (a) Micrograph of GFAP and BrdU stained astrocytes. Cells were starved for 28 h, treated for the last 24 h with 1 μM U69593 or 1 μM MOM-Sal-B and BrdU was added for the last 4 h of treatment. Primary Abs: mouse anti-BrdU monoclonal Ab (1 : 10 dilution) and rabbit anti-GFAP polyclonal Ab (1 : 1 dilution). Secondary Ab: Green fluorescence emitting fluorescein-conjugated anti-mouse IgG Ab (1 : 10 dilution) and Alexa Fluor 594 goat anti-rabbit IgG (1 : 700 dilution). Cells were designated type-1 or type-2 based on their morphology. Arrowheads show type-1 and arrows show type-2 astrocytes in the far right panel. (b) Effect of U0126 on U69593 signaling. Cells were starved 28 h, treated for the last 24 h with 1 μM U69593 ± 1 μM U0126 and BrdU was added for the last 4 h of treatment. In these studies, ≥ 1100 cells were counted for each determination; $n = 5$, $^{**}p < 0.01$ and $^{***}p < 0.001$ versus control; $^{†††}p < 0.001$ versus U69593 without inhibitor. (c) Effect of CD8-βARK-C on U69593 and MOM-Sal-B signaling. Cells were transfected with CD8 or CD8-βARK-C cDNA, starved 28 h, and treated for the last 24 h with 1 μM U69593 or 1 μM MOM-Sal-B. BrdU was added for the last 4 h of treatment. In these studies, ≥ 650 cells were counted for each determination; $n = 3$, $^{*}p < 0.05$ and $^{**}p < 0.01$ versus control; $^{†}p < 0.05$ and $^{††}p < 0.01$ versus their CD8 control in the presence of U69593. (d) Effect of β-arrestin 2 siRNA on U69593 and MOM-Sal-B signaling. Cells were transfected with KOR cDNA ± non-targeting or β-arrestin 2 siRNA. After 24 h on growth medium, they were starved for 28 h and treated for the last 28 h with 1 μM U69593 or 1 μM MOM-Sal-B. In these studies, ≥ 390 cells were counted for each determination; $n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus control; $^{†}p \leq 0.05$ and $^{†††}p < 0.001$ versus non-targeting siRNA control in the presence of U69593.

comparison of the two astrocytic culture systems suggests that even two highly related cell types can undergo changes in their cellular environment sufficient to alter temporal aspects of the downstream signaling targets such as that seen with KOR-mediated ERK phosphorylation. The KOR-selective antagonist norBNI also blocked MOM-Sal-B induced ERK1/2 activation in primary astrocytes (Fig. 5c) demonstrating the actions at KOR. However, unlike the results with immortalized astrocytes, norBNI did not completely abolish activity. Nevertheless, we did not observe a statistically significant change in ERK phosphorylation when using MOM-Sal-B in the presence of norBNI from the controls in the presence of norBNI alone ($p < 0.07$) Although unlikely, this partial inhibition may reflect either a need for higher concentrations of norBNI to completely eliminate MOM-Sal-B activity when using primary astrocytes or there may be a small KOR-independent action of MOM-Sal-B.



We then utilized the BrdU incorporation assay to assess cell growth in the primary cultures. Because of the presence of both type-1 and type-2 cells in the cultures generated under our growth conditions, GFAP immunocytochemical studies were performed on cells labeled with BrdU. As seen in Fig. 6a (far right panel), the primary astrocyte cultures were heterogeneous in that they contained GFAP-positive type-1 astrocytes that are flat polyhedral-shaped cells and type-2 that are spindle shaped and possess two or more processes (Raff *et al.* 1983). A small number of stellate-shaped GFAP-positive cells that contain many processes were also observed. Type-1 and type-2 cells were counted on the basis of their differences in morphology. The ratios of type-1 and 2 cells were generally about 1–3 : 1 in the P1 rat cortical astrocytes under our experimental conditions.

5′-Bromo-2′-deoxy-uridine labeling studies revealed that both U69593 and MOM-Sal-B stimulate proliferation in primary type-1 and type-2 astrocytes by two- to three-fold (Fig. 6b and c). Also the κ agonists elicited a greater increase in proliferation of primary type-1 and type-2 astrocytes than in immortalized type-1 astrocytes. The MAP kinase kinase inhibitor U0126 essentially abolished the proliferative actions of both κ agonists indicating that ERK1/2 mediates their activity. Both κ agonists elicit persistent ERK1/2 activation in these cells (Fig. 5a and b), consistent with the notion that sustained ERK1/2 activation is required for type-1 and -2 primary astrocyte proliferation.

CD8-βARK-C over-expression resulted in a complete inhibition of U69593 and MOM-Sal-B stimulation of primary type-1 and type-2 astrocyte proliferation (Fig. 6c). These results implicate Gβγ in the mechanism of κ agonist-induced proliferation of primary astrocytes. Furthermore, siRNA targeting β-arrestin 2 also abolished MOM-Sal-B elicited proliferation (Fig. 6d). Taken together, our findings suggest that the G protein and β-arrestin mediated signaling pathways are likely not additive and may be distinct.

Discussion

Here, we found that both κ opioid agonists, U69593 and MOM-Sal-B, activated ERK/MAPK via KOR in immortalized and primary astrocytes. Time course studies of ERK1/2 phosphorylation revealed that U69593 initiated a rapid (min) activation that was sustained for at least 2 h in immortalized and primary astrocytes. In contrast, MOM-Sal-B activated ERK1/2 transiently (< 30 min) in immortalized astrocytes but persistently in primary astrocytes. Nevertheless, both U69593- and MOM-Sal-B-induced ERK1/2 phosphorylation was inhibited by a Gβγ scavenger and β-arrestin 2 targeting siRNA. PTX inhibition of the early phase of ERK activation by U69593 supports the data gained with the Gβγ scavenger. In addition, the confocal microscopy data provides evidence that the agonists can induce β-arrestin 2–receptor interactions and together with the siRNA knockdown of β-arrestins 2 lends evidence to support β-arrestin 2-mediated signaling by KOR. Therefore, a multiplicity of κ opioid signaling pathways to ERK may exist in astrocytes.

As U69593 differs from MOM-Sal-B in its ability to induce sustained ERK activation in immortalized astrocytes, our results are also consistent with the notion of functional selectivity (Mukhopadhyay and Howlett 2005; for a review see Urban *et al.* 2007). Furthermore, the non-nitrogenous ligand is capable of selectively stimulating PTX-insensitive G protein and a transient β-arrestin 2-dependent signaling to ERK in immortalized astrocytes but it activates both G protein and sustained β-arrestin 2-dependent ERK signaling in primary astrocytes. However, in this instance, cellular environment may be a more important factor as MOM-Sal-B appears to be more functionally selective in immortalized than in primary astrocytes. This is keeping with recent signaling data demonstating unique binding properties of members of the salvinorin family (Groer *et al.* 2007; Vortherms *et al.* 2007; Tidgewell *et al.* 2008).

There is also precedent for μ opioid signaling to ERK via PTX-insensitive G proteins that is cell type specific (Belcheva *et al.* 2000). In COS-7 cells, [D-Ala2, Me-Phe4, Gly-ol5] enkephalin stimulates ERK phosphorylation via a PTX-insensitive mechanism, possibly entailing G(z) and G(12). In contrast, we have found that U69593 stimulation of ERK phosphorylation in an astrocytoma model (C6 glioma cells) is PTX sensitive whereas morphine is PTX insensitive (Barg *et al.* 1994; Bohn *et al.* 2000). This effect of cellular environment may be attributed to differences of ligand signaling in astrocytes versus neurons and in cells from different brain regions. In recent studies of κ opioid stimulation of c-Jun N-terminal protein (JNK) kinase phosphorylation in HEK293 cells, it was found that the elevation of phospho-JNK kinase by U50488 was PTX sensitive (Bruchas *et al.* 2007). The κ antagonist, norBNI also increased JNK kinase phosphorylation, but by a PTX-insensitive mechanism. As the authors point out, there is precedent for functional selectivity in which a ligand can display agonist and antagonist properties (see also Urban *et al.* 2007). However, the norBNI stimulation of JNK kinase phosphorylation was detected in both HEK293 cells and *in vivo*, thereby arguing against cell type specificity. Nevertheless, both our results and the norBNI data can be explained by G protein selectivity. The data shown here raise yet another parameter that must be considered in our attempts to understand functional selectivity, i.e. temporal aspects. The action of norBNI on JNK kinase phosphorylation was detected after 1 h treatment with the antagonist. It would be of interest to investigate the temporal profile of norBNI activation of JNK kinase. Moreover, in our studies and those of others, full understanding of functional selectivity will also depend upon delineation of

all the features of the individual pathways leading to activation of MAPKs. Differences in intermediary signaling components and the presence of endogenous receptor ligands in cells may impact on feedback mechanisms and ultimate outcomes.

Cortical astrogliosis has been found to be associated with neurodegenerative and other diseases such as autism, muscular dystrophy, human immunodeficiency virus, and dementia in drug users and Alzheimer's Disease (Bell *et al.* 1998; Terai *et al.* 2001; Yang *et al.* 2007; Buffo *et al.* 2008; Fatemi *et al.* 2008). This proliferative action may be triggered for the purpose of structural reorganization as proposed for spinal cord injury (Xu *et al.* 2007). Astrogliosis may be beneficial or it may be a maladaptive feature ensuing under pathophysiological conditions. Another factor that contributes to the complexity of the role of astrogliosis in injury and disease is the observed increase in dynorphin expression that also has neuroprotective as well as maladaptive roles (Hauser *et al.* 2005). This endogenous KOR ligand can promote DNA synthesis in developing brain at a time when astrocytes are being synthesized (Gorodinsky *et al.* 1995). Moreover, the astrogliosis seen after partial sciatic nerve lesion is not detected in dynorphin null mice (Xu *et al.* 2007). Therefore, the integration of the multiple-input ERK1/2 signaling pathways that contribute to cell proliferation is an important question to address.

The total abolishment of primary astrocyte proliferation driven by U69593 and MOM-Sal-B in the presence of U0126, CD8-βARK-C, or β-arrestin 2 targeting siRNA raises the issue of redundancy (Fig. 6). However, the considerable redundancies recently seen in receptor tyrosine kinase and phosphinositide 3 kinase signaling pathways in gliomas result in only partial blockade of viability and colony number (Stommel *et al.* 2007). We have implicated EGF receptor and phosphinositide 3 kinase in immortalized astrocytes (Belcheva *et al.* 2003, 2005) and partial inhibition of proliferation by CD8-βARK-C is evidenced in Fig. 5c. Since under similar conditions, CD8-βARK-C abolishes proliferation of primary astrocytes, it cannot be ruled out that both G protein and sustained β-arrestin 2 signaling play specific, integral roles required for the regulation of primary cell division. A recent report suggests that as many as 408 genes are involved in normal human fibroblast cell division (Bar-Joseph *et al.* 2008). Experiments are underway to delineate the mechanism further and to determine whether each pathway may affect an essential aspect of the complex process of primary astrocyte division.

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