

Research article

Delta Opioid activation of the Mitogen-activated protein kinase cascade does not require transphosphorylation of Receptor Tyrosine Kinases

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Abstract

Background: In this study, we investigated the mechanism(s) by which delta opioids induce their potent activation of extracellular signal-regulated protein kinases (ERKs) in different cell lines expressing the cloned δ -opioid receptor (δ -OR). While it has been known for some time that OR stimulation leads to the phosphorylation of both ERK isoforms, the exact progression of events has remained elusive.

Results: Our results indicate that the transphosphorylation of an endogenous epidermal growth factor receptor (EGFR) in the human embryonic kidney (HEK-293) cell line does not occur when co-expressed δ -ORs are stimulated by the δ -opioid agonist, D-Ser-Leu-enkephalin-Thr (DSLET). Moreover, neither pre-incubation of cultures with the selective EGFR antagonist, AG1478, nor down-regulation of the EGFR to a point where EGF could no longer activate ERKs had an inhibitory effect on ERK activation by DSLET. These results appear to rule out any structural or catalytic role for the EGFR in the δ -opioid-mediated MAPK cascade. To confirm these results, we used C6 glioma cells, a cell line devoid of the EGFR. In δ -OR-expressing C6 glioma cells, opioids produce a robust phosphorylation of ERK 1 and 2, whereas EGF has no stimulatory effect. Furthermore, antagonists to the RTKs that are endogenously expressed in C6 glioma cells (insulin receptor (IR) and platelet-derived growth factor receptor (PDGFR)) were unable to reduce opioid-mediated ERK activation.

Conclusion: Taken together, these data suggest that the transactivation of resident RTKs does not appear to be required for OR-mediated ERK phosphorylation and that the tyrosine-phosphorylated δ -OR, itself, is likely to act as its own signalling scaffold.

Background

Opioid receptors (ORs), like many other G protein-coupled receptors (GPCRs), are capable of signalling via the family of mitogen activated protein kinases (MAPKs). It has been postulated that activation of these kinases allows GPCR agonists to modulate such diverse molecular events as cell proliferation, differentiation, and survival [1]. To date, all three cloned opioid receptor types (μ , δ , κ) and the closely related nociceptin receptor have demonstrated the ability to signal through their heterotrimeric G proteins (G_i or G_o) to at least one type of MAPK [2-4]. Among the members of this family that are activated by opioids, are the two extracellular signal-regulated protein kinases (p44^{MAPK} (ERK 1) and p42^{MAPK} (ERK 2)) [5] and the p38 protein kinase [3]. However, the precise mechanism by which OR stimulation produces an increase in MAPK activity is still unknown and under investigation.

While receptor, cell, and tissue-specific differences almost certainly exist and appear to make any single mechanism of ERK activation unlikely, certain generalities have begun to emerge. For example, ERK activation by GPCRs is predominantly a Ras-dependent event, one that utilizes many of the upstream protein intermediates (i.e. Shc, Gab1, Grb2, mSOS, and MAPK kinase (MEK-1)) known to be used by single-transmembrane receptor tyrosine kinases (RTKs) like the epidermal growth factor receptor (EGFR) (for a review see [6]). When ERKs are activated after EGFR stimulation, an essential event is the sequential tyrosine phosphorylation of these intermediate proteins and their binding to the tyrosine phosphorylated EGFR before the GTP-loading of Ras. For the GPCR model, the tyrosine kinase(s) involved and the site of this multi-protein complex formation is less clear. For a number of GPCRs, including the lysophosphatidic acid (LPA) [7], β -adrenergic₂ (β_2 -AR) [8] and μ - and δ -OR receptors [9], the activation of a non-receptor tyrosine kinase of the Src or focal adhesion kinase (FAK) [10] families are involved. However, the question of what plasma membrane-spanning protein serves as the scaffold for Shc binding and beyond remains to be answered.

Two possibilities have emerged as potential sites of tyrosine phosphorylation and subsequent scaffold building in response to GPCR stimulation, which results in ERK activation: the GPCR, itself, or a co-expressed RTK (i.e. the EGFR) that would act as a surrogate. We and others and we have reported that μ - and δ -opioid receptors become tyrosine phosphorylated after agonist-stimulation [11,12]. Tyrosine phosphorylation of a membrane-bound protein is an essential step in ERK activation, because it creates SH2-binding domains that allow Shc and other proteins to associate into a multi-protein signalling complex. The mutation of one of the tyrosines (Y318F) in the δ -OR or the presence of the Src inhibitor, PP1, significant-

ly reduces tyrosine phosphorylation of this receptor and its ability to activate ERKs [11,13]. However, a small degree of δ -OR agonist-mediated ERK activation remained in cells expressing the Y318F mutant receptor, suggesting that a second mechanism or an additional tyrosine could be involved. These data suggest that the tyrosine phosphorylation of GPCRs may allow them to signal ERKs in the absence of RTK or FAK phosphorylation by acting as their own scaffolds.

However, the majority of the published studies have focused on the hypothesis that GPCRs must work in cooperation with RTKs (i.e. the EGFR) to produce ERK activation. Studies by Daub et al., [7] and Luttrell et al., [14] have demonstrated that stimulation of LPA ($G_{i/q}$ -coupled) and β_2 -ARs (G_s -coupled), respectively, results in the "transactivation /transphosphorylation" of co-expressed EGFRs. For example, both EPA and isoproterenol increase the tyrosine phosphorylation of the EGFR, which appears to be required for ERK activation since the presence of kinase-deficient EGFR mutants or the kinase inhibitor, AG1478, prevents both responses. It is hypothesized that trans-tyrosine phosphorylation of the EGFR creates protein phosphotyrosine binding sites that act as a surrogate scaffold for ERK activation [7]. Since ORs share many similarities with LPA and β_2 -ARs in the way they activate the ERK pathway [9,15], we chose to examine whether RTK transactivation occurs and is a necessary aspect of δ -opioid-mediated ERK activation.

Results

DSLET and EGF induce ERK phosphorylation in δ -OR-expressing HEK-293 cells

We have shown that δ -opioids increase the phosphorylation of ERK1/2 in HEK-293 cells when the mouse wild type (WT) δ -OR is stably expressed [11,13]. The concentrations required to observe DSLET-mediated ERK phosphorylation ($EC_{50} = 10$ nM; Figure 1a) are consistent with those reported for other δ -OR-mediated events, including G protein activation and adenylyl cyclase inhibition [16,17]. Moreover, no change in total ERK expression was observed, concomitantly, with ERK phosphorylation in any of our experiments (data not shown). Pretreating the cells with PTX (100 ng/ml), or the specific δ -OR antagonist, naltrindole (10 μ M), completely blocked the DSLET-induced increase in ERK phosphorylation (Figure 1b). These results suggest that DSLET utilizes a G_i -coupled δ -OR to stimulate ERK 1 and 2. We then took advantage of the HEK-293 cell line's endogenous expression of the EGFR to examine this receptor's ability to activate ERKs. Similar to DSLET, EGF (5 ng/ml) produces a robust activation of ERKs in δ -OR-expressing HEK-293 cells after a 5 minute treatment (Figure 2a). PTX did not attenuate EGF-mediated ERK activation (Figure 2a), although this response was completely blocked by the EGFR kinase inhibitor,

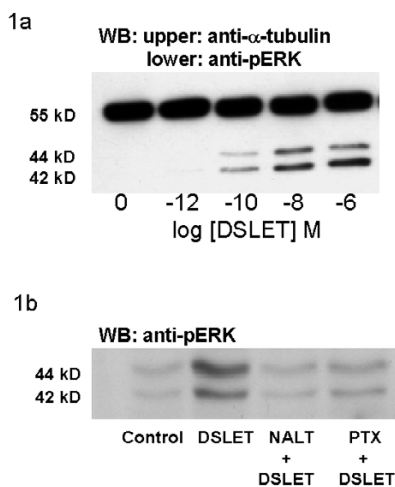


Figure 1
Western blots characterizing ERK phosphorylation by DSLET. (1A) HEK- δ -OR cells were serum-starved overnight prior to exposure to DSLET (1 pM – 1 μ M for 5 minutes). After DSLET exposure, cell lysates were prepared and ERK phosphorylation was determined by SDS PAGE, followed by immunoblotting of proteins onto nitrocellulose. Immunopositive bands correspond to α -tubulin, and the phosphorylated forms of ERK 1 and ERK 2. (1B) Inhibitory effects of naltrindole (10 μ M) or PTX (100 ng/ml) on DSLET-stimulated ERK phosphorylation. Naltrindole was added 60 minutes prior to DSLET, while PTX was added 18 hours before the δ -OR agonist. After DSLET exposure, cell lysates were prepared and ERK phosphorylation was determined by SDS PAGE, followed by immunoblotting of proteins onto nitrocellulose. Immunopositive bands correspond to the phosphorylated forms of ERK 1 and ERK 2. The immunoblots are representative of an experiment that was repeated three times.

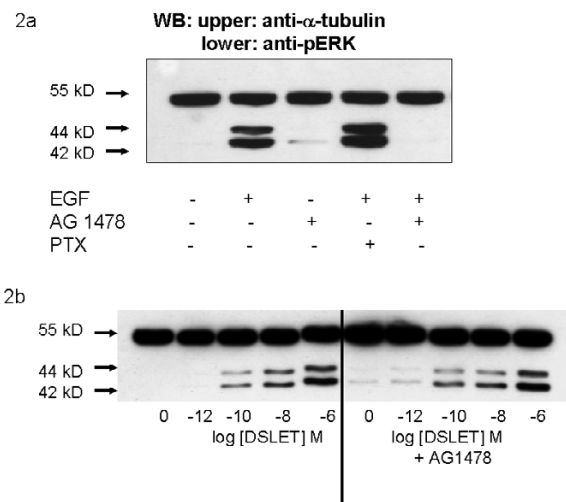


Figure 2
Western blots showing the effect of AG 1478 on ERK phosphorylation by EGF (2A) or DSLET (2B). (2A) HEK- δ -OR cells were serum-starved overnight prior to exposure to EGF (5 ng/ml for 5 minutes) in the presence or absence of PTX pretreatment (100 ng/ml; overnight) or AG1478 (1 μ M; 60 minutes prior). After DSLET exposure, cell lysates were prepared and ERK phosphorylation was determined by SDS PAGE, followed by immunoblotting of proteins onto nitrocellulose. Immunopositive bands correspond to α -tubulin, ERK 1, and ERK 2. (2B) AG1478 has no effect on DSLET-stimulated ERK phosphorylation. AG1478 (1 μ M) was added 60 minutes prior to DSLET (1 pM – 1 μ M for 5 minutes). After DSLET exposure, cell lysates were prepared and ERK phosphorylation was determined by SDS PAGE, followed by immunoblotting of proteins onto nitrocellulose. Immunopositive bands correspond to α -tubulin, ERK 1 and ERK 2. The immunoblots are representative of an experiment that was repeated three times.

AG1478 (1 μ M) (Figure 2a). Conversely, DSLET-induced ERK activation was unaffected by AG1478, suggesting that different tyrosine kinases are involved in opioid- and EGF-dependent MAPK activity (Figure 2b). DSLET-mediated ERK activation was significantly inhibited by the Src kinase inhibitor, PP1 (10 μ M) (Figure 3). PP1 also had a significant inhibitory (75% reduction) effect on MAPK activation by EGF (Figure 3).

DSLET-induced ERK activation does not produce hyperphosphorylation of the EGFR

Our next experiments were designed to examine whether the EGFR becomes tyrosine phosphorylated during ERK activation by DSLET. As stated in the introduction, several GPCRs (including the EPAR, β_2 -AR, and substance P (SP-R)) [14,18–20] increase EGFR phosphorylation during the course of ERK activation, and it is believed, that in these instances, this event is an initial step in the progression of the MAPK cascade. Our laboratory has previously

reported [11,13] that 5-opioids induce tyrosine phosphorylation of the δ -OR, and it appears that this is catalyzed by the non-receptor tyrosine kinase Src. However, transphosphorylation of the EGFR after OR activation has not been reported. WT- δ -OR-expressing HEK-293 cells were exposed to increasing concentrations of either DSLET (1–1000 nM) or EGF (0.1–100 ng/ml) for five minutes to induce ERK activation. As stated earlier, either DSLET or EGF increased the appearance of phosphorylated ERK in a time and concentration dependent manner. A portion of the lysates used to measure ERK activation was used to immunoprecipitate the EGFR or δ -OR to determine their degree of tyrosine phosphorylation. After immunoprecipitation of the FLAG-tagged δ -OR or the EGFR with the appropriate antibodies, we found that only DSLET induced tyrosine phosphorylation of the δ -OR, whereas only EGF potentiated EGFR phosphorylation (Figure 4). 1 μ M DSLET-induced tyrosine phosphoryla-

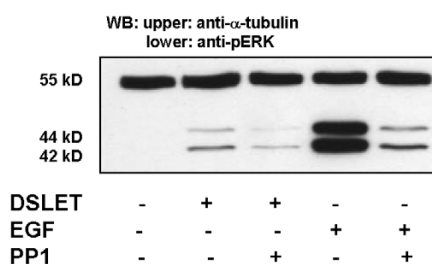


Figure 3

Western blot characterizing the effect of PP1 on ERK phosphorylation by EGF (5 ng/ml for 5 minutes) or DSLET (1 μM for 5 minutes). HEK-δ-OR cells were serum-starved overnight prior to exposure to EGF or DSLET. In some instances, cells were exposed to PP1 (10 μM) for 60 minutes prior to DSLET or EGF. After agonist exposure, cell lysates were prepared and ERK phosphorylation was determined by SDS PAGE, followed by immunoblotting of proteins onto nitrocellulose. Immunopositive bands correspond to α-tubulin, ERK 1, and ERK 2. The immunoblot is representative of an experiment that was repeated three times.

tion of the δ-OR was inhibited by naltrindole, PP1, but not by AG1478 (Figure 5). These results suggest that transactivation of the EGFR does not play a role in δ-OR phosphorylation or ERK activation by δ-opioids.

Determining whether a co-expressed RTK is structurally necessary for δ-OR-mediated ERK activation

EGFR down-regulation does not affect DSLET-mediated ERK activation in HEK-293 cells

Despite the lack of direct evidence that DSLET induces the transactivation of co-expressed RTKs, it is possible that the EGFR, in some way, contributes to ERK activation by opioids. It is possible that RTKs and GPCRs share important protein intermediates that are required during ERK activation. Therefore, we wished to determine what effects the down-regulation of the EGFR would have on ERK signaling via the δ-OR. HEK-293 cells expressing the WT-δ-OR were exposed to EGF (100 ng/ml) for 24 hours in order to down-regulate and desensitize the endogenous EGFR. After chronic EGF treatment, the cultures were washed with serum-free media and then exposed acutely (5 minutes) to either EGF (10 ng/ml) or DSLET (1 μM) to stimulate ERK phosphorylation. In EGF pre-treated cultures, acute EGF (10 ng/ml) was unable to increase the expression of phospho-ERKs compared to media-exposed controls (Figure 6). This lack of response appears to be due to the down-regulation of EGFRs from these cells as confirmed

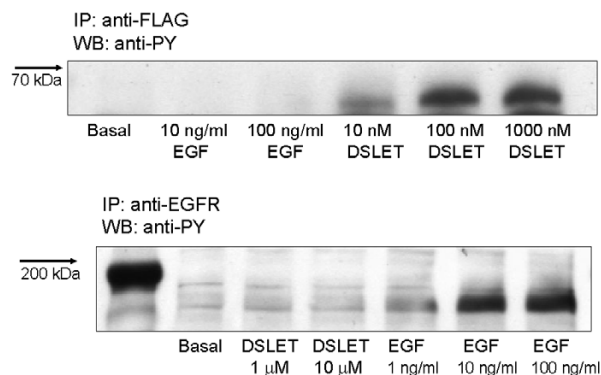


Figure 4

Western blots showing the effect of DSLET or EGF on tyrosine phosphorylation of the δ-OR or the EGFR. HEK-δ-OR cells were serum-starved overnight prior to exposure to EGF (0.1 – 100 ng/ml for 5 minutes) or DSLET (10 nM-10 μM for 5 minutes). After agonist exposure, cell lysates were prepared and the δ-OR or the EGFR was immunoprecipitated as described in the methods section using the appropriate antibodies. Tyrosine phosphorylation of these receptors was determined by SDS PAGE, followed by immunoblotting of proteins onto nitrocellulose. Immunopositive bands correspond to the tyrosine-phosphorylated forms of the δ-OR (upper panel) or the EGFR (lower panel). The immunoblots are representative of an experiment that was repeated three times.

by immunoblots using the anti-EGFR antibody (data not shown). However, DSLET-mediated ERK activation was unaltered in identically pretreated cultures.

In cells pretreated with DSLET (10 μM; 24 hours) to induce down-regulation of δ-ORs [21], DSLET (1 μM) was no longer able to activate the ERK cascade, but the ERK response to acute EGF (10 ng/ml) was unchanged compared to medium-treated controls (Figure 6). These results indicate that a reduction in the function and density of endogenous EGFRs has little effect on opioid-mediated MAPK cascades, and suggests that the enzyme cascades that control δ-OR- and EGFR-mediated ERK activation are separate.

DSLET activates ERK even in a cell line devoid of the EGFR

C6 glioma cells have been used as host cells for the transfection of several GPCR types, and have the advantage of expressing high levels of ERK, while being devoid of the EGFR. In C6 glioma cells that express the mouse δ-OR, DSLET produced a concentration-dependent increase in the appearance of phospho-ERK 1 and 2. DSLET-mediated ERK activation in C6-δ-OR cells was completely inhibited by either pre-treatment with PTX or naltrindole (data not shown). In contrast, EGF was unable to elicit ERK ac-

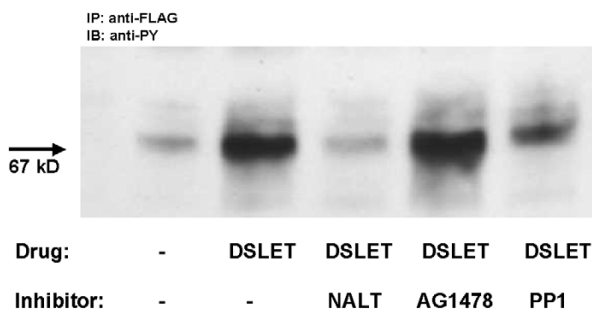


Figure 5

Western blot characterizing the effect of naltrindole, PPI, or AG1478 on tyrosine phosphorylation of the δ -OR by DSLET (1 μ M for 5 minutes). HEK- δ -OR cells were serum-starved overnight prior to exposure to DSLET. In some instances, cells were exposed to PPI (10 μ M) or AG1478 (10 μ M) for 60 minutes prior to DSLET. After agonist exposure, cell lysates were prepared and the δ -OR was immunoprecipitated as described in the methods section using the anti-FLAG antibody. Tyrosine phosphorylation of the δ -OR was determined by SDS PAGE, followed by immunoblotting of proteins onto nitrocellulose. Immunopositive bands correspond to the tyrosine-phosphorylated forms of the δ -OR. The immunoblot is representative of an experiment that was repeated three times.

tivation, at any concentration tested, up to 1 μ M (Figure 7). Furthermore, the EGFR antagonist, AG1478, had no effect on DSLET-mediated ERK activation, which eliminated the possibility that these antagonists produced non-specific, inhibitory effects (Figure 7).

Potential of endogenously expressed insulin receptors (IR) and PDGFR to act as surrogate protein scaffolds during ERK activation by δ -opioids

However, C6 glioma cells express additional RTKs including insulin (IR) and platelet-derived growth factor (PDGFR) receptors. It is possible that these sites can be used as surrogate scaffold proteins during ERK activation, and we explored the possibility that δ -opioids induce the transphosphorylation of these two RTKs. At concentrations of 10 μ M, neither DSLET, DADLE, nor DPDPE were found to increase the tyrosine phosphorylation of the IR or the PDGFR, whereas insulin (100 ng/ml) or PDGF (50 ng/ml) induced significant tyrosine phosphorylation of these sites (Figure 8). Moreover, we pre-treated C6- δ -OR cells with the IR antagonist, hydroxy-2-naphthalenylmethylphosphonic acid-tris acetoxymethylester (HNMPA-(AM)₃; 100 μ M), or the PDGFR antagonist, (tyrosphostin 9; 100 μ M) before the addition of DSLET (1 μ M). Despite the presence of these catalytic RTK inhibitors, δ -opioid-

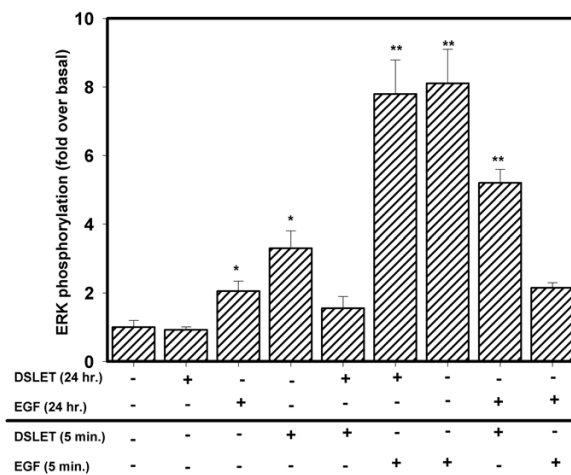


Figure 6

Effect of δ -OR or EGFR down-regulation on ERK phosphorylation by acute exposure to DSLET or EGF. HEK- δ -OR cells were serum-starved overnight prior to exposure to DSLET (10 μ M for 24 hours) or EGF (100 ng/ml for 24 hours) to induce receptor down-regulation. After chronic agonist pretreatment, cell monolayers were washed once with fresh media and exposed, acutely, to either DSLET (1 μ M for 5 minutes) or EGF (10 ng/ml for 5 minutes). After acute agonist exposure, cell lysates were prepared and ERK phosphorylation was determined by SDS PAGE, followed by immunoblotting of proteins onto nitrocellulose. PhosphoERK-immunopositive bands were quantified using the MCID system. Band intensities are expressed graphically (hatched bars) as: grain density \times band area - [region of detection (ROD)] \times pixel. Data points represent the mean \pm SEM of three experiments. * $p \leq 0.05$ compared to untreated samples and ** $p \leq 0.01$ compared to untreated samples as determined by ANOVA and the Tukey post-hoc test.

mediated ERK activation was not significantly altered in comparison to untreated cultures (Figure 9). Furthermore, immunoprecipitation of the δ -OR, followed by immunoblotting using anti-phosphotyrosine antibodies, revealed that these inhibitors did not attenuate tyrosine phosphorylation of δ -ORs after DSLET treatment (Figure 9). The results presented, herein, strongly suggest that transactivation of RTKs does not make a significant contribution to DSLET-induced ERK activation in at least two different cell lines.

Discussion

With the first evidence that GPCRs could signal via the MAPK cascade, many investigators have looked for a common mechanism of activation that could be applied across receptor types. Unlike the better understood mechanism whereby single-transmembrane RTKs (e.g. EGFR, IR, or PDGFR) stimulate ERKs [22,23], no single sequence of events appears to hold among GPCRs, even when they

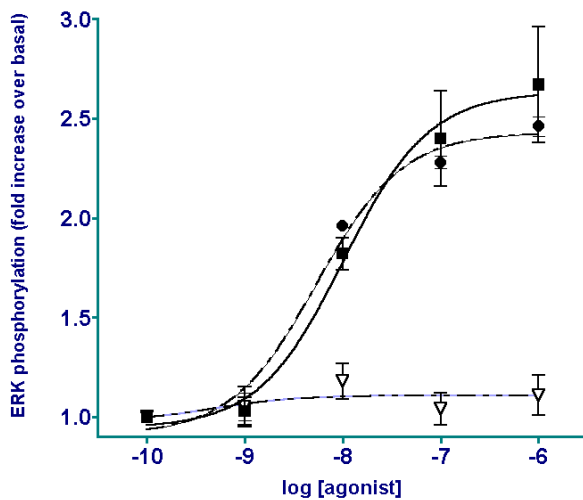


Figure 7

ERK phosphorylation by DSLET (filled squares) or EGF (open triangles) in C6 glioma cells stably-expressing the δ -OR. C6- δ -OR cells were serum-starved overnight prior to exposure to EGF (0.1 nM – 1 μ M for 5 minutes) or DSLET (0.1 nM – 1 μ M for 5 minutes). In some instances, DSLET-mediated ERK phosphorylation was performed in cells pre-treated with AG1478 (1 μ M; filled circles) 60 minutes prior to DSLET. After agonist exposure, cell lysates were prepared and ERK phosphorylation was determined by SDS PAGE, followed by immunoblotting of proteins onto nitrocellulose. PhosphoERK-immunopositive bands were quantified using the MCID system. Band intensities are expressed graphically as: grain density \times band area - [region of detection (ROD)] \times pixel. Data points represent the mean \pm SEM of three experiments.

are expressed in the same cell line. For instance, studies from two laboratories reported marked differences in the way the β_2 -adrenergic receptor (β_2 -AR) activates ERK 1 and 2 in HEK-293 cells. In one study, the β_2 -AR activates ERK through its coupling to a PTX-sensitive G_i protein and subsequent Ras activation [24], while, in another, ERK phosphorylation occurred in a PTX-independent and a G_s - and cAMP-dependent fashion [25]. Furthermore, these studies indicate that the same receptor may activate ERKs via one mechanism when it is endogenously expressed and via another when it is transfected into a cell line [25].

Despite numerous reported differences, it does appear that diverse GPCRs share common mechanistic themes during ERK stimulation, including the tyrosine-phosphorylation of Shc and its complexing with Grb2, mSOS recruitment, the GTP-loading of Ras, and Raf-1 activation leading to MEK-1 phosphorylation [1]. However, the progression of each of these downstream events after GPCR stimulation depends first on the tyrosine phosphorylation of a membrane-bound protein that will be used as a scaffold,

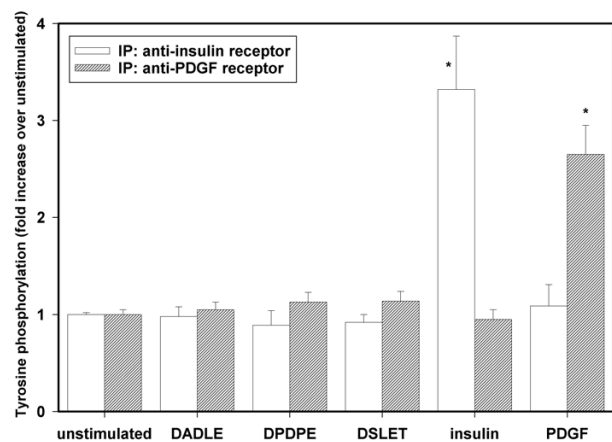


Figure 8

Effect of δ -OR agonists, insulin, or PDGF on tyrosine phosphorylation of the insulin receptor or the PDGF receptor. C6- δ -OR cells were serum-starved overnight prior to exposure to DADLE (10 mM), DPDPE (10 mM), DSLET (10 mM), insulin (100 ng/ml), or PDGF (50 mg/ml) for 5 minutes. After agonist exposure, cell lysates were prepared and the IR or the PDGFR was immunoprecipitated as described in the methods section using the appropriate antibodies. Tyrosine phosphorylation of these receptors was determined by SDS PAGE, followed by immunoblotting of proteins onto nitrocellulose. Immunopositive bands correspond to the tyrosine-phosphorylated forms of the IR (open bars) or the PDGF (hatched bars). Phosphotyrosine bands were quantified using the MCID system. Phosphotyrosine band intensities are expressed graphically as: grain density \times band area - [region of detection (ROD)] \times pixel. Data points represent the mean \pm SEM of three experiments. * $p \leq 0.05$ compared to untreated samples as determined by ANOVA and the Tukey post-hoc test.

fold, which will bind the other proteins (e.g. Shc, Grb2, Gab1, mSOS... etc...) [7]. Moreover, the exact identity of the tyrosine kinase(s) involved in this initial step appears to vary among receptor types, with RTKs, non-receptor protein tyrosine kinases (PTKs), or FAKs being involved in particular instances [26].

In this report, we questioned whether the EGFR acts as a surrogate scaffold during ERK activation through the δ -OR, as it appears to do for several other GPCRs [7]. To act as a surrogate-binding site for complex formation, the literature states that this protein must first become tyrosine-phosphorylated [7]. Tyrosine phosphorylation of the EGFR is apparent after agonist stimulation of the α_2 -AR (COS-7), β_2 -AR (COS-7), insulin-like growth factor (IGF-1; COS-7), substance P, and the lysophosphatidic acid receptor (LPA) [7]. In addition, each of the aforementioned ligands also increases ERK phosphorylation when

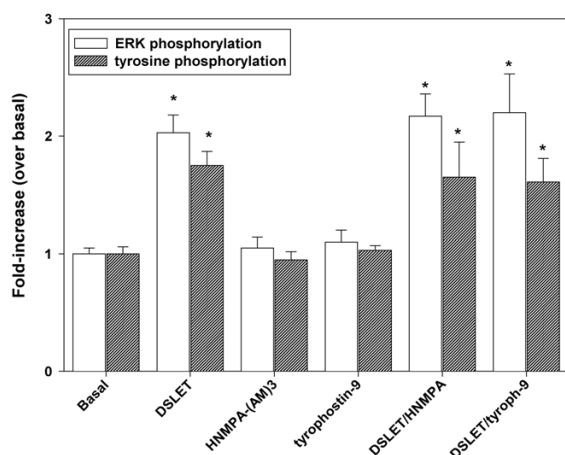


Figure 9

Effect of antagonists to the insulin receptor (HNMPA-(AM)₃) or the PDGF receptor (tyrosphostin-9) on ERK phosphorylation and tyrosine phosphorylation of the δ -OR by DSLET. C6- δ -OR cells were serum-starved overnight prior to exposure to DSLET (1 μ M for 5 minutes). In some instances, DSLET exposure was performed in cells pre-treated with HNMPA-(AM)₃ (100 μ M) or tyrosphostin-9 (100 μ M) 60 minutes prior to the δ -OR agonist. After agonist exposure, cell lysates were prepared and divided into aliquots for the analysis of ERK phosphorylation or tyrosine phosphorylation of the δ -OR. The δ -OR was immunoprecipitated as described in the methods section using the appropriate antibodies. Tyrosine phosphorylation of the δ -OR or ERK phosphorylation was determined by SDS PAGE, followed by immunoblotting of proteins onto nitrocellulose. Immunopositive bands corresponding to the tyrosine-phosphorylated form of the δ -OR (hatched bars) or ERK phosphorylation (open bars) were quantified using the MCID system. Band intensities are expressed graphically as: grain density \times band area - [region of detection (ROD)] \times pixel. Data points represent the mean \pm SEM of three experiments. * $p \leq 0.05$ compared to untreated samples as determined by ANOVA and the Tukey post-hoc test.

the proper receptor is expressed in these cell lines. ERK activation and EGFR phosphorylation via each of these receptors was inhibited by prior exposure to the EGFR kinase inhibitor, AG1478.

However, the Src inhibitor, PP1 or the expression of a dominant-negative Src mutant, will reduce GPCR-mediated transphosphorylation of the EGFR and GPCR-mediated ERK activation to varying degrees (partial to total), suggesting that at least two different tyrosine kinases can be involved [18]. Src was found to co-immunoprecipitate with the phosphorylated EGFR in COS-7 cells exposed to either LPA or EGF [14]. These authors reported that the autophosphorylation of Y350 in the EGFR does not occur

after LPA exposure, suggesting that c-Src is the principal tyrosine kinase in this instance and not the EGFR's intrinsic tyrosine kinase. Src has also been found to co-immunoprecipitate with the agonist-stimulated β_2 -AR during the period of ERK activation [8]. Our previous work [11,13] suggested that a Src-like protein tyrosine kinase was primarily responsible for tyrosine phosphorylation of the δ -OR and ERK activation induced by DSLET when the receptor was expressed in CHO or HEK-293 cells, because both responses were blocked by the presence of 50 μ M PP1. The seemingly dominant role of Src in opioid-mediated ERK stimulation is strengthened in the present report because of considerable evidence that the EGFR antagonist, AG1478, was unable to reduce DSLET-mediated ERK activation. This suggests that the EGFR has minimal, if any, involvement in the opioid-activated ERK cascade. It is possible, that the dual coupling of the LPAR to both inhibitory (G_i) and stimulatory ($G_{12/13}$ and G_q) G proteins is a reason for these differences.

In this report, we have presented considerable evidence that δ -ORs and EGFRs activate ERKs, independently, without the need for transphosphorylation of a co-expressed RTK. We used C6 glioma cells, transfected to stably express the mouse δ -OR, to examine ERK activation by δ -opioids in a line that does not express the EGFR to confirm our results obtained in the receptor down-regulation experiments. We observed that DSLET activated p42^{mapk} and p44^{mapk} very robustly in this cell line. C6 glioma cells do express growth factor receptors that bind PDGF and insulin. However, neither of these proteins has been reported to act as a surrogate scaffold during ERK activation by non-opioid GPCRs [27]. In the present report, DSLET-mediated ERK phosphorylation was unaltered by the presence of the IR or PDGFR antagonists, HNMPA-(AM)₃ and tyrosphostin 9, respectively. It is probable that the δ -OR in C6 glioma cells also acts as its own platform for the binding of Shc, etc., since the δ -OR also undergoes agonist-mediated tyrosine phosphorylation in this particular cell line (Kramer et al., unpublished observations), similar to what we previously reported for the δ -OR expressed in Chinese hamster ovary or HEK-293 cells [11,13]. Thus, in at least two different cell lines, HEK-293 and C6 glioma, we have significant evidence that transactivation of an endogenously expressed RTK is not required for ERK activation through opioid receptors.

While the EGFR does not appear to be phosphorylated during DSLET-mediated ERK activation, it is possible that the EGFR is somehow involved in this response when it is present. Buist et al., [27] reported that endogenously-expressed LPA and endothelin-1 (ET-1) receptors in SK-N-MC cells do not activate ERKs unless the EGFR is co-expressed, although each GPCR is functional as evidenced by their ability to increase intracellular [Ca^{2+}]. In the pres-

ence of the EGFR, ligand activation of LPA and ET-1 receptors did not increase EGFR phosphorylation even though both the LPA and ET-1 receptors stimulated ERK activity by 10-fold over basal [27]. Specifically, key protein intermediates may be physically associated with endogenous RTKs in HEK-293 cells, and therefore, down-regulation of the EGFR may negatively affect ERK phosphorylation by opioids. To examine this, we induced significant down-regulation of EGFR density and function by exposing δ -OR-HEK-293 cells to 100 ng/ml EGF for 24 hours. Functionally, these cells are no longer capable of activating ERK 1 or 2 in response to 10 ng/ml EGF (Figure), whereas this concentration produces a strong effect in non-pre-treated cells. Conversely, DSLET-mediated ERK phosphorylation is actually greater in chronically-treated EGF cultures to that observed in cells not previously exposed to high EGF concentrations. This may represent a compensatory effect to counteract the loss of function of an important growth factor receptor. In a similar experiment, a 24-hour exposure to DSLET (10 μ M) induced significant δ -OR down-regulation and an almost complete loss of DSLET-mediated ERK phosphorylation. MAP kinase activation by a 5 minute exposure to EGF was unaffected by δ -OR down-regulation. These results, taken together, suggest that GPCRs and RTKs can act as independent regulators of MAP kinase cascades, although interactions between the two receptor types may exist in certain cases.

In conclusion, we report that the δ -OR agonist, DSLET, induces significant ERK activation in the absence of any increase in phosphorylation of the co-expressed EGFR in HEK-293 cells. HEK-293 cells endogenously express the EGFR, and EGF produces robust ERK activation concomitant with EGFR tyrosine phosphorylation. Immunoprecipitation studies showed that the δ -OR becomes tyrosine phosphorylated after exposure to DSLET, but not to EGF. These studies were repeated in δ -OR-transfected C6 glioma cells, which do not express the EGFR. In these cells, DSLET was still able to induce ERK activation. Additional studies showed that the two RTKs, which are present in C6 glioma cells, IRs and PDGFRs, had no apparent role on ERK activation by a δ -opioid agonist. Furthermore, inducing down-regulation of either the EGFR or δ -OR by chronic agonist treatment has little effect on ERK activation by the other receptor in HEK-293 cells, suggesting that these pathways are distinct. These results demonstrate that opioid-mediated ERK activation occurs independently of EGFR transphosphorylation, and that the tyrosine-phosphorylated δ -OR may serve as the primary scaffold.

Materials and Methods

Drugs and compounds

All chemicals used for these studies were of reagent grade and obtained from the following sources. The Src inhibitor, 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo

[3,4-*d*]pyrimidine (PP1), was purchased from Calbiochem (La Jolla, CA). The EGFR antagonist, AG1478 (4-(3-chloroanilino)-6,7-dimethoxyquinazoline), the insulin receptor antagonist, hydroxy-2-naphthalenylmethylphosphonic acid-tris acetoxymethylester (HNMPA-(AM)₃), and the PDGFR antagonist, tyrospostin, 9 were purchased from Biomol (Plymouth Meeting, PA). The opioid agonist, [D-Ser-Leu-enkephalin-Thr (DSLET), was received from the National Institute of Drug Abuse (NIDA, Baltimore, MD) and the antagonist, naltrindole, was purchased from Research Biochemicals, Inc., (Natick, MA). Pertussis toxin (PTX) was purchased from Sigma Chemical Co. (St. Louis, MO). Insulin was obtained from University Hospital of New York University and PDGF was purchased from Biomol (Plymouth Meeting, PA). All other compounds were obtained from the sources listed under the individual methods.

Cell transfection and culture

C6 glioma cells were stably transfected with the cDNA encoding the epitope-tagged (FLAG) mouse δ -opioid receptor (C6- δ OR) and were created in our laboratory using the Fip-In transfection method. HEK-293 cells were transfected, in house, with the cDNA encoding the FLAG-tagged wild-type (HEK- δ OR) mouse δ -OR. Complete methods for the stable transfection of HEK-293 cells to express the wild type receptor can be found in a previous report [13]. Both cell lines were seeded in 6-well dishes at an initial cell density of 1×10^6 cells/well and were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) supplemented with 2 mM L-glutamine, 5% fetal bovine serum (FBS) (Gemini Bio-Products, Calabasas, CA), and 100 μ g/ml gentamycin sulfate (Gemini Bio-Products, Calabasas, CA). Selection pressure was maintained by the presence of 1 mg/ml G418 sulfate (Life Technologies, Inc.) in the culture media, which was kept at 37°C in 7% CO₂/humidified air and was changed every 3–4 days.

Cell treatment and the immunoprecipitation (IP) of the δ -OR or the EGFR

C6- δ OR or HEK- δ OR cells were not treated until they reached 80–90% confluency. Relative levels of receptor expression were continuously measured among cell types by radioligand binding assays [28] to insure that all cells used expressed 1–2 pmol/mg protein of ³H-diprenorphine (³H-DIPR; ³H-DIPR, 54 Ci/mmol, Amersham; 0.1–1.5 nM) binding. Sixteen hours before the addition of drugs, the culture medium was removed and replaced by fresh serum-free DMEM. All inhibitors/antagonists were added at least thirty minutes before opioid agonists or EGF. After a five-minute opioid or EGF incubation, the cell monolayers were rinsed twice with ice cold phosphate-buffered saline (PBS), and the cells were solubilized by the addition of 300 μ l of solubilization buffer (50 mM Tris-HCl/Base (pH7.4), 1 % Triton X-100, 10% glycerol,

1.5 mM MgCl₂, 1.0 mM CaCl₂, 300 mM NaCl, 1 mM NaVO₃, and the protease inhibitors leupeptin (100 µg/ml), PMSF (100 µM), and aprotinin (100 µg/ml) for 60 minutes at +4°C. Soluble proteins were isolated by centrifugation at 30,000 g for ten minutes at +4°C. Primary cell lysates were analyzed for protein concentration using the Bio-Rad method. A mouse monoclonal antibody raised against the FLAG-epitope (Flag-M₁ antibody (2–5 µg/µl), Sigma Chemical, St. Louis, Mo.) was used to immunoprecipitate the tagged δ-OR from the cell lysates, and a murine anti-EGFR antibody ((5 µg/µl) clone LA22; Upstate Biotechnology, Lake Placid, NY) was used to immunoprecipitate out the EGFR. Antibodies to the insulin (C-19 epitope-β subunit; rabbit polyclonal IgG (5 µg/µl)) and PDGF receptor (C-20 epitope; rabbit polyclonal IgG (5 µg/µl)) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Immunoprecipitation of all receptor types was performed using magnetic Dynabeads (DynaL Bioscience, Lake Success, NY) coated with anti-mouse or anti-rabbit IgG and used according to the manufacturer's instructions. Proteins were prepared for SDS-PAGE by boiling them in 2 × Laemmli's sample buffer and stored at -70°C until used.

Immunoblotting for the presence of phosphorylated MAPK (ERK1/2) and phosphorylated tyrosines

SDS PAGE and immunoblotting (IB) were performed following the methods contained in a previous report [11], and all procedures were performed at room temperature unless otherwise noted. Proteins (5–20 µg/lane) were separated by using 10–20% acrylamide gels, and electroblotted onto nitrocellulose membranes. Non-specific binding sites were blocked by incubating the nitrocellulose for 60 minutes in blocking buffer (25 mM Tris-HCl/Base, 150 mM NaCl, 0.05% Tween-20, and 3% nonfat dry milk). For the identification of the activated form of ERK (pERK) from primary cell lysates, we used an antibody raised against pERK-1/2 (1:5,000 dilution in blocking solution, Santa Cruz Biotechnology, Santa Cruz, CA). Total MAPK levels were quantified using the ERK-1 antibody (C-16 epitope; 1:5,000 dilution in blocking solution), which was also obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibodies raised against phosphorylated tyrosines (clone PY20 (1:2,000 dilution), Santa Cruz Biotechnology, Santa Cruz, CA) were used to identify tyrosine phosphorylated EGFR or δ-ORs. Incubation with all primary antibodies was carried on overnight at +4°C. Membranes were then incubated for 60 minutes in the presence of a goat-anti-mouse secondary antibody linked to horseradish-peroxidase (1:2,000) at room temperature. Immunoreactive proteins were visualized using a horseradish peroxidase-sensitive ECL chemiluminescent Western blotting (WB) kit (Pierce).

Data Analysis and statistical methods

Radioligand binding curves (to determine k_D and B_{max}) were generated, and all regression analyses were performed using the LIGAND curve-fitting program [29]. Graphs were produced using Sigmaplot for Windows (version 4.0). Images of immunoreactive bands were captured on x-ray film and analyzed using the MCID morphometric system (Imaging Research Inc., Ontario, Canada). All immunochemical assays were performed at least three times, although results from a single experiment are sometimes shown for clarity. One-way and two-way analyses of variance (ANOVA) and the post-hoc Tukey test were used for multiple comparisons at a minimum significance level of $p \leq 0.05$. Student's t-test was substituted for the ANOVA for simple two-sample tests at the same significance level. Statistical data are expressed as mean ± standard error (SE) of the indicated number of observations.

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