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Opioid control of MAP kinase cascade

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Abstract

Activation of G protein-coupled receptors (GPCRs) may result in phosphorylation of extracellular signal-regulated kinases 1/2 (ERK 1/2). The signaling pathway involves ectodomain shedding, generating epidermal growth factor (EGF)-like ligands, which in turn stimulate the mitogen-activated protein kinase (MAPK) via EGF receptors. The present study investigates into the control of MAPKs by opioidergic GPCRs in human embryonic kidney cells (HEK 293). Experiments were conducted with cells expressing opioid receptors, G protein-coupled receptor kinases, and ERKs. The outcome of our studies let us suggest that EGF-like ligands released by opioid receptor stimulation utilize different EGF receptors to phosphorylate ERKs, while EGF utilizes type 1 receptors. Differences between multiple opioid receptors are apparent with respect to the activation of ERKs. EGF rapidly triggers internalization of the fluorescent EGF receptor type 1, but we failed to observe any sequestration of this receptor type upon exposure of cells to an opioid, since opioids most likely trigger stimulation of a different EGF receptor type. In conclusion, G protein-coupled opioid receptors control the MAPK cascade in a similar fashion as described for non-opioid GPCRs, although distinct differences exist between μ-, δ- and κ-receptors. EGF-induced ERK activation is mediated by EGF receptor type 1 while opioid receptor activation seems to brings about stimulation via EGF receptor type.

Keywords: MAP kinase; Opioid; Epidermal growth factor receptor; Human embryonic kidney 293 cell; Extracellular signal-regulated kinase; G protein-coupled receptor

1. Introduction

Activation of G protein-coupled receptors (GPCRs), including the opioid receptors, transduces extracellular signals into an array of intracellular responses as heterotrimeric G proteins dissociate. The liberated Gα and Gβγ subunits stimulate a variety of effectors and trigger the translocation of cytosolic proteins. More recently, the growth factor receptors, representing receptor tyrosine kinases, received increasing attention, since GPCRs have been shown to activate growth factor receptors which in turn accounts for phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK 1 or $p44_{MAPK}$ and ERK 2 or $p42_{MAPK}$) (Shah and Catt, 2004). The ERKs represent one of three major mitogen-activated protein kinase (MAPK) classes, which are activated by GPCRs via receptor tyrosine kinases such as the epidermal growth factor (EGF) receptor or the fibroblast growth factor receptor (Leserer et al., 2000). Indeed, one of the best studied examples of receptor cross-talk concerns GPCRs and EGF receptors, which eventually results in the phosphorylation of distinct ERK pools of the cytoplasm. These activated ERK pools may regulate nuclear transcription, affect the function of membrane components or phosphorylate cytoplasmatic and cytoskeletal substrates (Luttrell, 2003). However, the cellular mechanisms activating the MAPK cascade are known to exhibit major variations between GPCR types, individual cell systems, or various permanent cell lines (Luttrell, 2002).

Evidence accumulated during the past that opioid receptors cross-talk to EGF receptors most likely via Gα- and Gβγ-subunits released from $G_{i/o}$ or possibly from G_q (Burt et al., 1996; Kramer et al., 2000, 2002; Schmidt et al.,

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2000). In analogy to the activation of MAPKs by non-opioid GPCRs it appears likely that one signaling pathway for the activation of ERKs by opioid receptors involves metalloproteases, consisting of matrix metalloproteases (MMPs) and enzymes that contain a disintegrin and a metalloprotease domain (ADAM). Each MMP- and ADAM-family consist of several individual enzymes owning the capacity to cause ectodomain shedding of soluble ligands, e.g. the heparin-binding EGF (HB-EGF) of the EGF family (Raab and Klagsbrun, 1997). HB-EGF is processed from a membrane-located precursor and activates ERKs by means of EGF receptors (Prenzel et al., 1999; Pierce et al., 2001). However, little is known about how opioid receptors bring about stimulation of EGF receptors. One hypothesis suggests that EGF receptors become transactivated by metalloproteases-caused release of EGF receptor-like ligands, e.g. HB-EGF, in a protein kinase C (PKC)-dependent manner (Belcheva et al., 2002). With respect to non-opioid GPCRs there is strong evidence that these receptors activate PKC, which contacts and subsequently stimulates the cytoplasmatic tail of metalloproteases, resulting in release of EGF receptor-like ligands (Luttrell, 2003). Another notion favours the activation of ERK-pools by scaffolding proteins, such as the cytosolic arrestins (Luttrell, 2002, 2003). These reflections stimulated the present study, focusing on multiple opioid receptors and how they account on EGF receptorcontroled cascade of ERK/MAPK activation in human embryonic kidney (HEK) 293 cells.

2. Materials and methods

2.1. Chemicals

The biochemical reagents were of analytical grade and purchased from Calbiochem (Deisenhofen, Germany) and Sigma (Taufkirchen, Germany). Deltorphin II was from Bachem (Heidelberg, Germany), morphine–HCl from Merck (Darmstadt, Germany), sufentanil from Janssen Pharmaceuticals (Beerse, Belgium), etorphine–HCl from National Institute of Drug Abuse (USA), and naloxone from Dupont (Geneva, Switzerland). U50488H (*trans*(±)-3,4dichloro-*N*-methyl-*N*-[2(1-pyrrolidinyl)cyclohexyl]benzeneacetamide) was from Sigma (Taufkirchen, Germany).

Epidermal growth factor, galardin, PP2 (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolol[3,4-*d*)pyrimidine), Ro 31-8425 (bisindolylmaleimide–HCl), chelerythrine, and bisindolylmaleimide were from Calbiochem.

2.2. Cell culture reagents

Chemicals were purchased from Gibco (Karlsruhe, Germany), fetal calf serum and Dulbecco's modified Eagle's medium (DMEM) were from PAN (Nürnberg, Germany), and cell culture material was from NUNC (Wiesbaden, Germany).

2.3. Antibodies

The anti-$EGFR_{human}$-antiserum (Ab-3) was directed against the ligand binding site of the receptor and was employed according the recommendations of manufacturer (Merck Biosciences, Schwalbach, Germany). The anti-phospho p44/p42 MAP kinase antibody was purchased from New England Biolabs (Frankfurt, Germany) and used according to the company's instructions.

2.4. Cell culture

HEK 293 cells were maintained in DMEM supplemented with 10% fetal calf serum as described. Experiments were conducted with cells at 60% confluency.

2.5. Construction of expression vectors

The general procedure for the construction of fusion proteins is described previously (Schulz et al., 1998), using the Clontech (Palo Alto, USA) vectors pEGFP, encoding the red-shifted variant of wt GFP (enhanced GFP, EGFP), and pDsRed. The green fluorescent EGFR represents the type 1 receptor (Gene Bank Accession No. X00588).

2.6. Cell transfection

Cells were transfected with "Effectene Reagent" (Qiagen, Hilden, Germany), and were used for experiments 48 h after transient transfection. Stable transfections of cells to express opioid receptors were conducted according to standard procedures, using G418 selection.

2.7. Western blots

Cells raised in six-well-plates were kept in DMEM for 2 h in the absence of fetal calf serum, and thereafter challenged with the ligand under investigation. After 5 min the medium was substituted by 350 μl Lämmli-buffer, heated (95 °C) and 25 μl of each well was submitted to gel electrophoresis. Proteins were electroblotted to nitrocellulose at 4 °C, and incubated with the anti-phospho-antibody overnight at 4 °C. After appropriate secondary antibody steps (1 h, 25 °C, 1:20.000) and rinsing, the blots were developed with enhanced chemiluminescence detection.

2.8. Gelatin zymography

MMP activity of opioid-exposed HEK 293 cells, expressing δ-opioid receptors (HEK/DOR), were analyzed by zymography as described by Tanimura et al. (2002). Briefly, HEK/DOR cells grown on 24-well plates were incubated in serum-free medium for 2 h, and subsequently exposed to indicated opioids in absence or presence of naloxone (10 μM) for 1 h at 37 °C. Thereafter, medium (200 μl) was removed and mixed with 1/5 volume of non-

reducing sample buffer (312 mM Tris–HCl, 10% sodium dodecyl sulfate (SDS), 0.1% phenol red, pH 6.8). Proteins were dissolved by electrophoresis on 8% polyacrylamid gel, containing 0.1% gelatin. Gels were washed with buffer (50 mM Tris–HCl, 1% Triton X-100, pH 7.5) for 1 h to remove SDS, and were subsequently incubated in reaction buffer (50 mM Tris–HCl, 1% Triton X-100, 10 mM $CaCl_2$, 150 mM NaCl, 0.01% NaN_3) for 24 h at 37 °C. Gels were stained with 0.2% Coomassie blue in 30% methanol, 10% acetic acid for 1 h at room temperature. Destaining of digested gelatin was conducted with 30% methanol in 10% acetic acid. Gelatinolytic activity by opioid-induced release of MMPs is indicated by unstained band on a blue background.

2.9. Confocal microscopy

The technique employed is detailed by Schulz et al. (1999).

3. Results

3.1. Activation of MMPs by opioid receptors

The EGF receptor, a tyrosine kinase receptor, is activated by GPCRs, utilizing membrane-associated EGF-like factors, e.g. HB-EGF (Riese and Stern, 1998). The release of these ligands requires an activation of MMPs located mainly at the outer cell surface. To examine the effect of opioids on MMPs we employed the gelatin zymography method (Tanimura et al., 2002). This technique takes advantage of the ability of gelatin to bind Coomassie blue. Since GPCRs are expected to induce liberation of MMPs into the extracellular millieu that is, the culture medium of δ-opioid receptors (DORs) carrying HEK293 cells, we challenged the cells by an opioid and submitted the medium to gel electrophoresis. MMPs separate between 50 and 100 kDa, where they will digest gelatin. The gelatin-free areas of the gel lose their Coomassie blue and become visible on a dark blue gel. Fig. 1A discloses that the medium from deltorphin-stimulated HEK/DOR cells submitted to gelatin zymography results in Coomassie blue free bands. These unstained bands coincide with MMPs exhibiting a molecular weight of 66 kDa (MMP 2), indicating proteolytic activities of opioid-released proteases. Beside the liberation of MMP 2, weak proteolytic activity was observed in the range of 90 kDa where MMP 9 is expected to migrate (data not shown). The MMP activity is blocked by galardin, an inhibitor of MMP types 1, 2, 3, 8, and 9, and by the metalloprotease inhibitor batimastat, which also attenuates the activities of ADAMs (Wolfsberg et al., 1995; Moss et al., 2001). It was proposed that proteases of the ADAM group account for proteolytic liberation of HB-EGF, and ADAM-9 (Prenzel et al., 1999), -12 (Asakura et al., 2003) and -17 (Gschwind et al., 2001) have been implicated in PKC-dependent HB-EGF shedding triggered by GPCRs. In general, the findings observed here for δ-receptor activation extents to HEK cells expressing μ- and κ-opioid receptors (data not given). Moreover, the opioid-induced release of proteases is antagonized by naloxone (Fig. 1B). Our results clearly reveal that galardin suppresses the opioid-induced gelatinolytic activity, which is entirely in line with the action of other GPCRs on ectodomain shedding of EGF ligands (Prenzel et al., 1999).

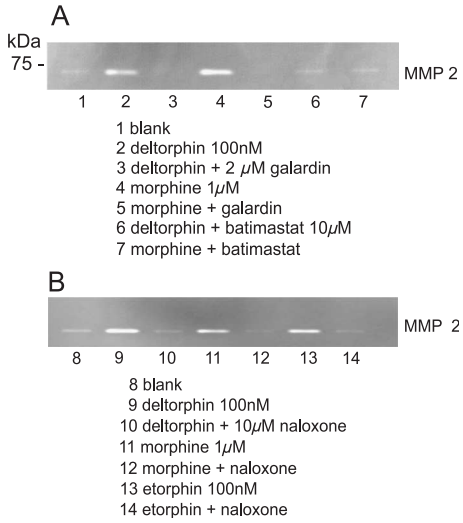

Fig. 1. Gelatin zymography of incubation medium from HEK cells expressing δ-opioid receptors. The gelatinolytic activity of opioid-released metalloproteases manifests at 66 kDa (matrix metalloprotease 2) and 86 kDa (MMP 9). (A) Minimal enzymatic activity is observed in absence of any opioid (blank). Exposure of cells to deltorphin (lane 2) reveals a strong gelatinolytic activity, which is blocked by the MMP inhibitor galardin. Analogous results were obtained with morphine. Lanes 6 and 7 document an inhibition of MMP-liberated enzymatic activity by batimastat. (B) The gelatinolytic activities induced by deltorphin, morphine and etorphine, respectively, are completely blocked by preincubation of cells with naloxone. The opioid antagonist itself did not release enzymatic activity (data not given). The results documented (A and B) represent at least six independent experiments each.

3.2. Phosphorylation of ERKs by δ-opioid receptors

The opioid-induced MMP activation, that is ectodomain shedding of ligands transactivating EGFRs, caused us to examine whether galardin and possibly batimastat prevent the activation of the MAPK cascade by opioids in HEK/DOR cells expressing exogenous opioid receptors. HEK/DOR cells were exposed to opioids in the absence and presence of a metalloprotease inhibitor and were subsequently submitted to Western blotting for determining ERK 1/2 phosphorylation. Fig. 2A reveals that activation (phosphorylation) of ERK 1/2 by deltorphin is attenuated by the protease inhibitors galardin and batimastat, respectively. Remarkably, inhibition of ERK phosphorylation requires a preexposure of cells with at least 10 μM batimastat for 2 h. This finding corroborates with data obtained with muscarinic GPCRs (Prenzel et al., 1999) that

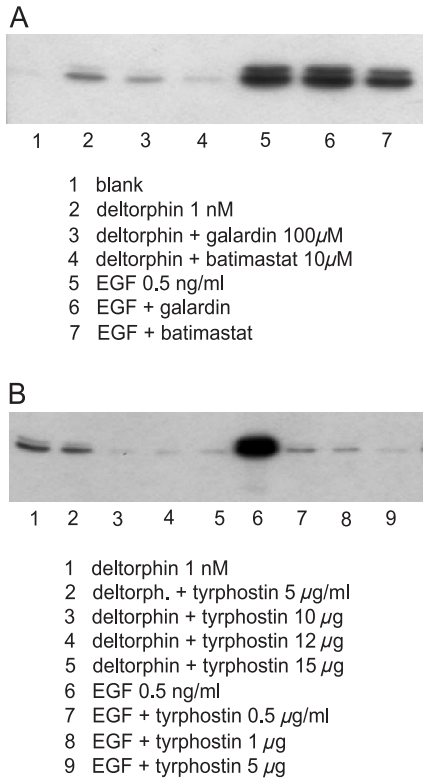

Fig. 2. Detection of phosphorylated extracellular signal-regulated kinase (ERK) 1/2 by electrophoresis and phospho-specific antibodies. Phosphorylation was triggered by exposure of HEK/DOR cells to deltorphin and epidermal growth factor, respectively. The upper bands represent ERK 1, the lower ERK 2. (A) Deltorphin-induced ERK phosphorylation (lane 2) is reduced by preincubation of cells with galardin (lane 3) and batimastat (lane 4). Both MMP inhibitors fail to block phosphorylation brought about by EGF. (B) Inhibition of deltorphin- and EGF-induced phosphorylation of ERK 1/2 by tyrphostin. While inhibition of deltorphin-caused ERK phosphorylation requires about 10 μg tyrphostin per ml incubation medium, inhibition of EGF-induced ERK phosphorylation is observed with already 0.5 μg tyrphostin per ml. The presented data stand for at least four independent experiments.

batimastat in lower concentrations failed to affect ERK phosphorylation. As expected, both galardin and batimastat did not disturb the activation of ERKs by EGF (Fig. 2A). The results support the notion that opioid receptors induce MAP kinase phosphorylation via ectodomain shedding of ligands transactivating EGF receptors (Raab and Klagsbrun, 1997). This concept may be supported by the action of tyrphostin, a highly specific blocking agent of EGF receptors (Levitzki and Gazit, 1995), as the drug blocks phosphorylation of ERKs induced either by deltorphin or by EGF (Fig. 2B). However, the concentration of tyrphostin (0.5 μg/ml) required to block EGF-triggered phosphorylation is about 20-fold lower (10 μg/ml) as compared to that required to block ERK activation initiated by an equipotent δ-opioid receptor stimulation, using deltorphin. This finding may indicate that EGF and EGF-like ligands released by the opioid utilize different EGF receptor types (Raab and Klagsbrun, 1997).

The results with opioids are in line with the general notion that liberation of EGF-like ligands activates EGF receptors, which exert a key function for GPCR-controlled MAPK cascade. This view would be strengthened when the access of opioid released EGF-like ligands to EGF receptors is prevented. These conditions should result in an attenuation of ERK 1/2 phosphorylation. To attack this issue we employed an anti-EGF receptor-antibody directed against the ligand binding site of the EGF receptor, which should prevent access of EGF or EGF-like ligands to their receptors. The Western blot (Fig. 3) exhibits the effect of EGF and of deltorphin on ERK 1/2 phosphorylation in HEK/DOR cells in absence and presence of the EGF receptor-antibody. The EGF receptor-antibody (2 μg/ml medium) was shown to clearly eliminate the effect of EGF on ERK phosphorylation. Under similar experimental conditions, but using instead of EGF an equieffective deltorphin concentration, the antibody failed to block the opioid-induced phosphorylation of ERK. Since a maximal activation of ERKs is observed within few minutes after opioid challenge, the failure of the anti-EGFR-antibody cannot be explained by a delayed release of EGF-like ligands (Raab and Klagsbrun, 1997). Noteworthy is the poor capacity of etorphine to activate ERKs via δ-receptors. Most likely opioids bring about ectodomain shedding of HB-EGF, and the EGF-like domain of the liberated molecule is believed to activate EGF receptors (Carpenter and Wahl, 1991). However, at least four human EGF receptor types exist (Plowman et al., 1993). EGF activates the receptor type 1 (Carpenter et al., 1979), while HB-EGF activates EGF receptors 1 and 4 (Higashiyama et al., 1992). The multiplicity of EGF receptors and their ligands (Higashiyama et al., 1992) suggests that the anti-EGF receptor-antibody employed in this study is directed against the type 1 receptor, as it blocks the action of EGF, but is not directed against type 4 receptor, which is activated by HB-EGF. Thus, the different concentrations of tyrphostin required to inhibit ERK phosphorylation by either EGF or opioids strongly support the view that EGF and EGF-like ligands activate different EGF receptor types.

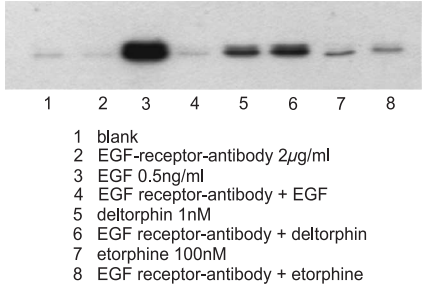

Fig. 3. The effect of anti-EGF-receptor-antibody on phosphorylation of ERK 1/2. While the antibody itself fails to affect ERK phosphorylation, 0.5 ng EGF/ml strongly phosphorylates ERKs. Preincubation of HEK/DOR cells with the antibody blocks EGF-induced ERK phosphorylation, but is without effect on ERK phosphorylation caused by deltorphin or etorphine. Results represent five independent experiments.

The concept that EGF and opioid-liberated EGF-like ligands utilize distinct EGF receptor types to phosphorylate ERK 1/2 was further strengthened by experiments with HEK/DOR cells desensitized to EGF. Fig. 4 reveals that the cells become completely desensitized to EGF when exposed to 100 ng EGF per ml medium for 24 h, as judged by the ability of EGF to phosphorylate ERKs. Interestingly, stimulation of EGF-desensitized cells with deltorphin not only completely retained their potency to phosphorylate ERK 1/2, but even elevated ERK activation. This finding indicates that prolonged EGF exposure did not affect those receptors mediating deltorphin-induced ERK phosphorylation. Tyrphostin did not lose activity to block phosphorylation of ERKs by deltorphin in EGF-desensitized cells. On the other hand, prolonged exposure of HEK/DOR cells to morphine (1 μM, 24 h) does induce tolerance to deltorphin-induced ERK phosphorylation, but the effect of EGF was left undisturbed (data not given). Together, our findings suggest distinctly different pathways for EGFand for opioid-induced signaling to bring about MAPK activation. We propose that multiple EGF receptors account for these differences.

3.3. Multiple opioid receptors and MAP kinase signaling

Activation of δ-opioid receptors has been reported to phosphorylate ERK 1/2 (Kramer et al., 2002). This finding was confirmed here and extended to μ- and κ-receptors. Again, the tyrphostin concentration required (Fig. 5) to block EGF-induced phosphorylation of ERK1/2 is lower as compared to that necessary to block ERK activation by deltorphin (1 nM, HEK/DOR), sufentanil (100 nM, HEK/MOR), or U50488H (1 nM, HEK/KOR). The outcome of these experiments further support the view that multiple opioid receptors use different types of EGF receptors to activate ERKs.

Opioid receptors were shown here to phosphorylate ERKs via EGF receptors. This notion challenges the view by Kramer et al. (2002) reporting, that phosphorylation of ERKs triggered by δ-opioid receptors in HEK/DOR cells is not blocked by tyrphostin. The authors concluded therefore that transactivation of EGF receptors does not play a role for

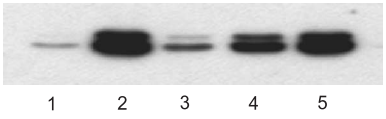

Fig. 4. Treatment of HEK/DOR cells with EGF (100 ng/ml, 24 h) desensitizes the cells to the phosphorylating action of EGF on ERKs. In contrast, deltorphin not only retained its phosphorylating action on ERKs in EGF-desensitized cells, but even increased its activity. Data stand for five independent experiments.

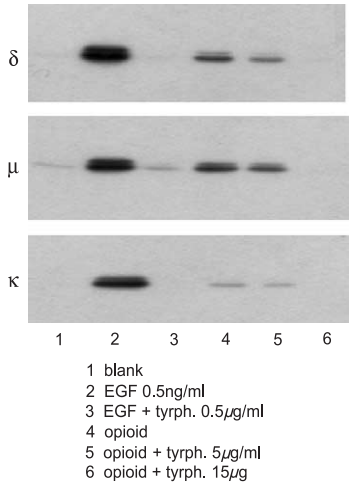

Fig. 5. Antagonism of EGF- and opioid-induced ERK phosphorylation by tyrphostin in HEK cells expressing δ-, μ- or κ-opioid receptors. The opioids employed for ERK activation were deltorphin (1 nM, 5 min, 37 °C), sufentanil (100 nM, 10 min, 37 °C) and U50488H (1 nM, 5 min, 37 °C). EGF stimulation of HEK cells strongly phosphorylated ERKs, which was blocked by 0.5 μg tyrphostin per ml. ERK phosphorylation by δ-, μ- or κ-opioid receptors was wiped out by 15 μg tyrphostin per ml (lane 6). Data represent three independent experiments.

ERK activation by δ-opioid receptor agonists. Moreover, they suggested that EGF receptors in general possess no function with respect to the opioid-activated MAPK cascade. This interpretation by Kramer et al. conflicts with our findings, demonstrating an activation of EGF receptors by μ-, δ- and κ-receptors, respectively.

Transactivation of EGF receptors is further induced by activation of the non-receptor tyrosine kinase c-Src (Zwick et al., 1999). This enzyme phosphorylates EGF receptors, and inhibitors of the Src family attenuate GPCR-induced activation of EGFRs (Bokemeyer et al., 2000). Here we show that the c-Src kinase inhibitor PP2 (Salazar and Rozengurt, 1999) alleviates ERK 1/2-induced phosphorylation by δ- as well as by κ-opioid receptors, being in line with findings by Kramer et al. (2002) regarding the δ-opioid receptor. However, PP2 proved inactive to affect ERK phosphorylation brought about by μ-receptors (Fig. 6).

Protein kinase C (PKC) is likely to mediate activation of MAPK triggered by certain GPCRs (Hawes et al., 1995; Luttrell, 2003). While phorbol myristate acetate causes PKC-dependent HB-EGF domain shedding involving ADAM 9 (Gechtman et al., 1999), HB-EGF cleavage induced by activation of G_i-coupled receptors neither utilizes PKC nor ADAM 9 protease (Prenzel et al., 1999). We thus examined the action of three PKC inhibitors on opioid-triggered ERK phosphorylation. HEK cells expressing δ-, μ- and κ-receptors, respectively, were incubated with Ro 31-8425 (up to 10^{-6} M, 1 h), chelerythrine (10^{-6} M, 1 h) or bisindolylmaleimide (10^{-7} M, 30 min) prior to deltorphin (δ-receptor, 10^{-9} M), sufentanil (μ-receptor, 10^{-7} M, 10 min), or U 50488H (κ-receptor, 10^{-9} M)

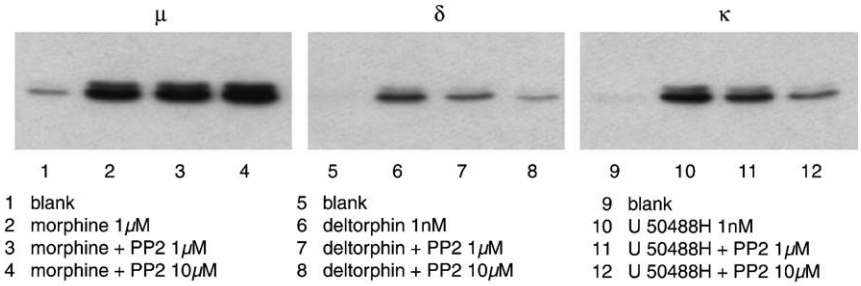

Fig. 6. Effect of PP2, an inhibitor of non-receptor tyrosine kinase c-Src, on opioidinduced phosphorylation of ERKs. HEK cells were transfected to express μ-, δ- and κ-receptors, respectively, and preincubated (2 min) with PP2. While PP2 failed to inhibit ERK phosphorylation caused by morphine (μ-receptor agonist), it reduced phosphorylation by deltorphin (δ-receptor) and by U50488H (κ-receptor). Data represent three independent experiments each.

exposure (5 min). Western blot studies revealed that none of the compounds tested affected the ability of opioids to phosphorylate ERK 1/2 (data not given). We conclude, therefore, that opioids do not utilize PKC for stimulation of the MAPK cascade. However, the phorbol ester was proposed to mediate activation of ERKs (Beguinot et al., 1985). Our experiments confirmed this finding, demonstrating by means of Western blotting an activation of ERKs by the phorbol ester (1 μM, 2 h; data not given). This action of the phorbol ester was still observed, although less pronounced, after 24 h pretreatment of cells with the ester.

3.4. Internalization of EGF receptors: a confocal microscopy study

Activation of non-opioid GPCRs may cause internalization of EGF receptors (Grewal et al., 2001). Since G protein-coupled opioid receptors activate EGFRs, we aimed to test by means of confocal microscopy whether cross-talk between opioid receptors and EGF receptors causes internalization of EGF receptors. For this reason EGF receptors type 1 were fused with Enhanced Green Fluorescence Protein (EGFP), and these constructs were transiently expressed in HEK cells transfected to stably express δ-opioid receptors. Further experiments were conducted with HEK cells coexpressing in addition G protein-coupled receptor kinase 3 (GRK 3) fused with DsRed (Schulz et al., 2002). GRK-DsRed fusion proteins were included as control, since activated opioid receptors cause translocation of cytosolic GRKs towards the cell membrane (Schulz et al., 2002). The experimental conditions selected here allow monitoring of EGF receptor-EGFP migration in live HEK cells.

We first tested whether activation of EGF receptors by deltorphin causes internalization of the fluorescent EGF receptor-EGFP chimera. HEK/DOR cells coexpressing EGFP-labeled EGF receptors and GRK3-DsRed were challenged with the δ-receptor ligand. The images of Fig. 7 disclose that δ-receptors rapidly induce accumulation of cytosolic GRK3-DsRed at the cell membrane (1 min post drug challenge), indicating that the overexpressed δ-receptors and the GRK-DsRed constructs were functional. However, deltorphin failed to cause internalization of EGF receptors as no generation of green fluorescent vesicles were detected during the observation period (20 min). Opioid concentrations of even up to 1 μM were unable to bring about any visible internalization of green fluorescent EGF receptors, regardless of the duration of cell exposure to the agonist (up to 24 h). However, deltorphin induces interruption of the opioid signal transmission within seconds after receptor activation. We can thus not rule out that this mechanism does account for the failure of the opioid to internalize EGF receptor-EGFP constructs. Employing HEK cells expressing μ- and κ-opioid receptors, respectively, we also failed to observe internalization of EGF receptors. However, these findings together with our Western blot studies (see above) are in line with the notion that internalization of EGF receptors is not required for ERK 1/2 phosphorylation (DeGraff et al., 1999).

The effect of EGF on internalization of EGF receptor-EGFP fusion proteins was tested in HEK cells coexpressing the tagged EGF receptor and GRK3-DsRed. EGF challenge caused the formation of green vesicles, indicating an internalization of EGF receptors (Fig. 8). Receptor migration led to an accumulation of vesicles in the cytoplasma within 45 min, resembling a time course reported for opioid receptors (Schulz et al., 1999, 2002). When monitoring the fate of cytosolic GRK3-DsRed after EGF challenge it appears that no translocation of the kinase was observed the first 10 min thereafter. Subsequently, GRK3-DsRed was observed to accumulate in the same location where fluorescent EGF receptors formed clusters. We assume, therefore, that EGF receptor carrying vesicles recruit cytosolic GRK3. EGF-stimulated internalization of EGF receptors was completely blocked by tyrphostin (15 μg/ml, data not given). The efficient internalization of EGF receptors reported here contrast a recent report that HEK cells poorly internalize EGF receptor-GFP constructs (Carter and Sorkin, 1998).

Activation of GPCRs forces ERK 1/2 into the cell nucleus where the kinases phosphorylate transcription factors (Pearson et al., 2001). We examined by confocal microscopy whether opioid receptors mediate a similar effect. C-terminus EGFP-labeled ERK 2 was employed to

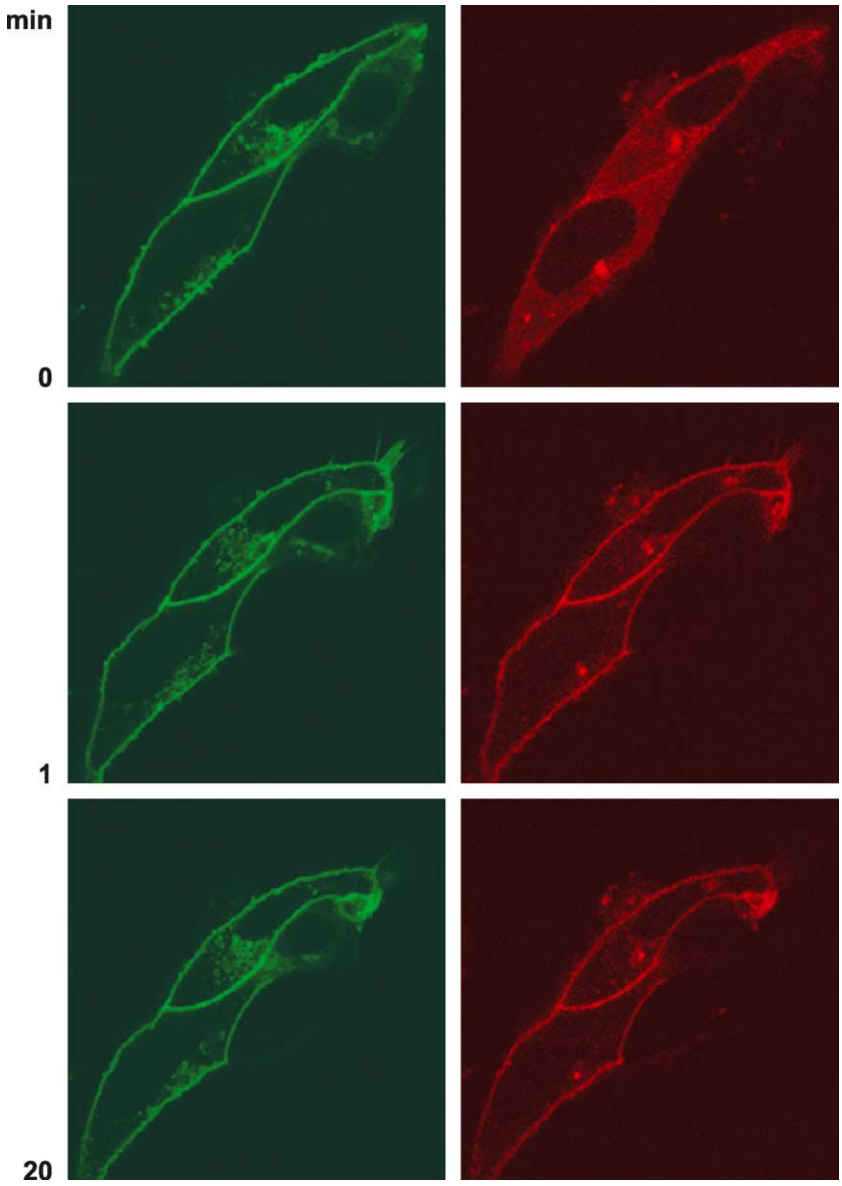

Fig. 7. Confocal microscopy studies with live HEK/DOR cells coexpressing EGFRs fused with enhanced green fluorescence protein (EGFP) and G protein-coupled receptor kinase 3 (GRK 3) fused with red fluorescent DsRed. Stimulation of cells with deltorphin (200 nM) immediately caused translocation of GRK3-DsRed towards the cell membrane (1 min), but failed to trigger the formation of green fluorescent vesicles, indicating internalization of EGFP-tagged EGF receptors (observation period 20 min). Data represent 10 independent experiments.

transfect HEK/DOR cells. Fig. 9 discloses the time course of translocation of fluorescent ERK 2. Transfection of cells results in a wide spectrum of ERK-EGFP expression, that is some cell nuclei fluoresce very bright, allowing no recognition of fluorescence translocation from cytoplasm to the nucleus.

The less efficiently transfected cells exhibit minor fluorescence in the nucleus and even less in the cytoplasma prior to drug challenge (0 min). Exposure of those cells to deltorphin results in a rapid translocation of fluorescence from the cytoplasma to the nucleus (Fig. 9, 1 and 2 min) and redistribution follows thereafter (15 min). The settings of the confocal microscope were left unchanged during the time course of the experiment, allowing a direct comparison of green fluorescence intensity in cytoplasm and nucleus during the different stages after opioid challenge. Deltorphin-induced translocation and redistribution of ERK-EGFP was completely blocked by naloxone as well as by tyrphostin (data not given). The experiment clearly indicates that deltorphin-induced translocation of ERK is mediated by δ-opioid receptors (naloxone antagonism) and involves EGF receptors (tyrphostin blockade). This finding adapts to the general concept that GPCRs, including δ-opioid receptors, own the capability to control the ERK/MAP kinase cascade (Leserer et al., 2000).

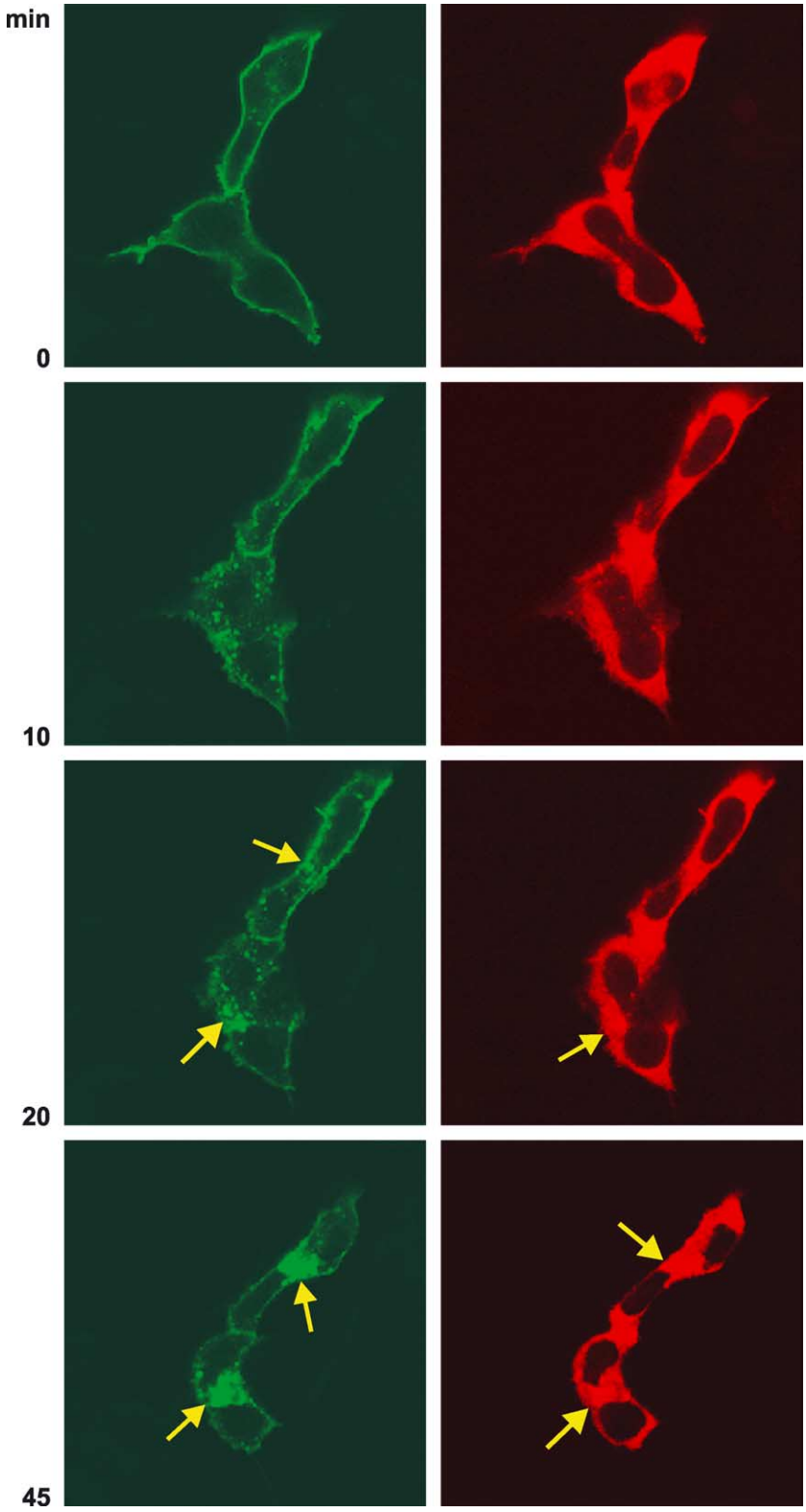

Fig. 8. Internalization of EGFRs following stimulation with EGF. Confocal microscopy imaging of HEK cells coexpressing EGFP-tagged EGF receptors and GRK3-DsRed show a strict association of green fluorescent receptors with the cell membrane and an allocation of GRK3-DsRed with the cytoplasma, sparing the nucleus (0 min). EGF challenge caused the formation of green vesicles (10 min), while GRK3-DsRed was unchanged. 20 min after EGF exposure green vesicles started to form clusters (arrows), and first signs of red fluorescence accumulation (arrow) was observed. Forty-five minutes after EGF stimulation vesicles carrying EGFRs-EGFP gathered in the cytoplasma (arrows), which was accompanied by GRK3-DsRed (arrows). Data represent five independent experiments.

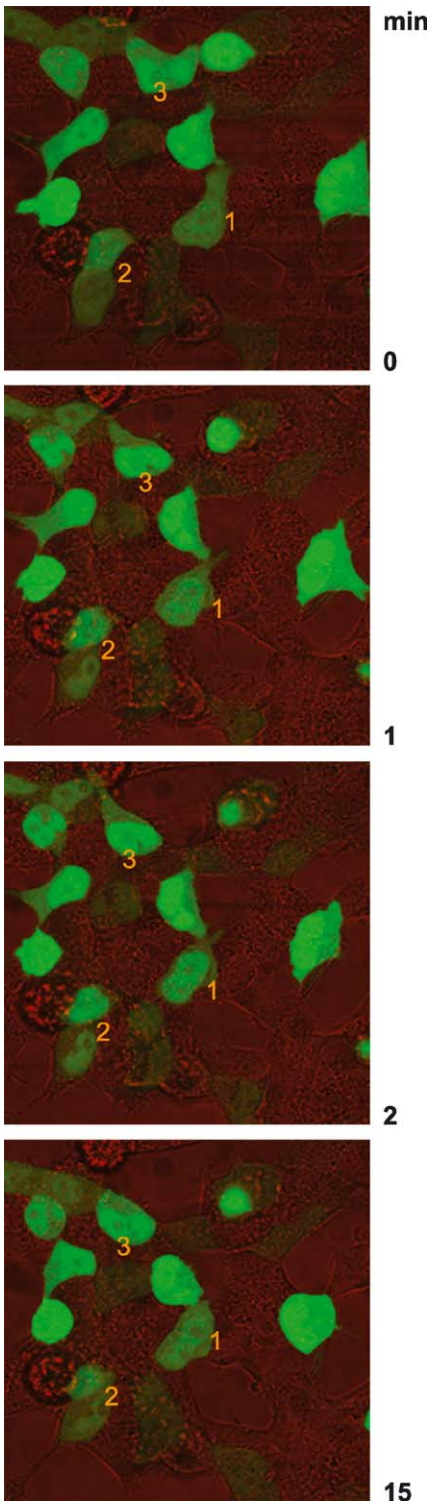

Fig. 9. Confocal microscopy study of live HEK/DOR cells expressing EGFP-tagged extracellular signal-regulated kinase 2. Prior to stimulation of cells with deltorphin (200 nM) green fluorescence was found in the nucleus and to a lesser degree in the cytoplasm (0 min; cells 1, 2 and 3). One minute after opioid exposure fluorescence intensity increased in the cell nucleus, which was even more apparent after 2 min. Numbers inside the confocal images indicate identical cells during the observation period. Redistribution of green fluorescence from the cell nucleus towards the cytoplasm is observed 15 min after EGF challenge. Data given represent 12 independent experiments.

4. Discussion

The outcome of the present investigation supports the notion that the underlying mechanisms activating the MAP kinase cascade by GPCRs, including the opioid receptors, are very similar. Opioids were found to induce ectodomain shedding, as they trigger activation of MMPs made visible by gelatinolysis, and inhibitors of metalloproteases reduce the activation of ERKs. Regardless of the opioid receptor type stimulated, although with different intensities, ERK phosphorylation occurred in HEK cells via transactivation of EGF receptors.

A major outcome of our studies suggests that EGF and opioids utilize different EGF receptor types to phoshorylate ERKs. This concept rests on several findings. First, anti-EGF receptor-antibodies block EGF-induced activation of ERK 1/2 but not opioid-triggered ERK phosphorylation. Second, chronic exposure of HEK/DOR cells with EGF desensitizes the action of EGF but not that of opioids. Third, blockade of EGF-mediated ERK 1/2 phosphorylation requires about 20-fold less tyrphostin than found for the inhibition of opioid-induced ERK phosphorylation. These results are best explained by proposing distinctly different EGF receptors responsible for the phosphorylation of ERK 1/2 caused by EGF and by opioids. Information is available that EGF activates EGF-receptors type 1, and the EGF-like ligand HB-EGF, liberated by metalloproteases, binds to EGF-receptor type 1 and 4 (Raab and Klagsbrun, 1997). With respect to the EGF-antibody used in this study we assume its action on EGF receptors type 1, since the immunoglobulin failed to prevent access of the deltorphin-released EGF-like ligand to EGF receptor type 4 (Higashiyama et al., 1992). Both EGF- and opioid-induced pathways for ERK phosphorylation, utilizing the suggested receptor type 1 or type 4, appeared differently sensitive to tyrphostin. The notion that multiple EGF receptors account for EGF- and for HB-EGF-triggered ERK phosphorylation is strengthened by the outcome with EGF-desensitized HEK/DOR cells. The literature provides numerous examples documenting that development of tolerance to a defined receptor system leaves iso-receptors unaffected or even increases their capacity or affinity (Johnson and Fleming, 1989) as observed here for the opioid-controled ERK activation in EGF-desensitized cells.

An unresolved question relates to the mechanism how opioid receptors control metalloproteases and subsequently the activation of ERKs. GPCRs were proposed to activate cytosolic PKC which contacts the cytosolic segment of metalloproteases, causing in turn cleavage of HB-EGF (Leserer et al., 2000). Our studies with opioid receptors and several PKC inhibitors do not support this notion. An alternative concept assigns a mediatory function to Gβγ subunits (Luttrell, 2003) and to arrestins (DeFea et al., 2000). Both pathways have not been investigated in the present study. However, there is increasing evidence that arrestins recruit Src-kinases. These enzymes are stimulated

by Giα and Gβγ subunits, causing the non-receptor tyrosine Src kinases to phosphorylate EGF receptors without activation of their intrinsic tyrosine kinase (Bokemeyer et al., 2000; Zwick et al., 1999). We showed here that the Src kinase inhibitor PP2 (Salazar and Rozengurt, 1999) alleviated opioid-induced activation of ERK 1/2. This effect on δ-receptor-induced ERK phosphorylation required 10^{-5} M PP2, which resembles previous studies with GPCRs and PP1, also a c-Src inhibitor (Kramer et al., 2002).

Our confocal microscopy studies with living cells revealed an efficient capability of HEK cells to internalize EGF receptors upon exposure to EGF, resembling the fate of opioid receptors during the process of internalization (Schulz et al., 2002). We found it of interest, although we cannot offer an explanation, that vesicles carrying internalized EGF receptor-EGFP chimera recruit GRK3-DsRed with a delay of at least 15 min after EGF-exposure of HEK cells. However, we never observed an internalization of the EGFP labeled EGF receptor following opioid challenge of HEK cells expressing exogenous opioid receptors. Apparently, since the fluorescent EGF receptor employed in this study represents the type 1 receptor, and opioids may stimulate the EGFR type 4, no formation of vesicles carrying green fluorescent EGF receptors would be expected.

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