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Potentiation of G-Protein-Coupled Receptor-Induced MAP Kinase Activation by Exogenous EGF Receptors in SK-N-MC Neuroepithelioma Cells

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Lysophosphatidic acid (LPA) and endothelin-1 (ET-1), two ligands for G-protein coupled receptors (GPCRs), induce activation of mitogen activated protein kinase (MAPK). Surprisingly, LPA and ET-1 did not induce MAPK activation in SK-N-MC neuroepithelioma cells, even though these GPCR ligands evoked a rapid, transient rise in intracellular free Ca^{2+} concentration in these cells, indicating that SK-N-MC cells express functional LPA- and ET-1-receptors. Transient transfection of the EGFR into SK-N-MC cells, which do not express endogenous EGFR, potentiated LPA- and ET-1-induced MAPK activation. LPA and ET-1 did not enhance basal level tyrosine phosphorylation of the transfected EGFR in SK-N-MC cells. Even though the mechanism of LPA- and ET-1-induced MAPK activation in EGFR-transfected SK-N-MC cells remains to be determined definitively, our results provide strong evidence that the EGFR links these GPCRs to MAPK activation.

Two classes of receptors have been identified that mediate activation of the Ras/Mitogen Activated Protein Kinase (MAPK) pathway in response to extracellular signals, receptor protein-tyrosine kinases (PTKs) and heterotrimeric G-protein coupled receptors (GPCRs) (1). It has been established that recruitment of the guanine nucleotide exchange factor for the Ras GTPase, Son of Sevenless, to the membrane is the activating step in the mechanism of receptor PTK-mediated activation of MAPK. In this respect relatively little is known about how GPCRs couple to MAPK.

Lysophosphatidic acid (LPA) is the ligand for a putative GPCR and it is the prototypic GPCR agonist that activates the Ras/MAPK pathway (2). The LPA receptor couples to at least three distinct heterotrimeric G proteins, G_q, $G_{12/13}$ and G_i. Pertussis toxin-sensitive G_i induces Ras.GTP accumulation, eventually leading to MAPK activation, which is mediated by a PTK (3). The identity of the PTKs that are involved in GPCR-induced MAPK activation is currently under debate.

Candidate PTKs that have been identified to link GPCRs to MAPK include cytoplasmically localized PTKs, Src, Lyn, Syk, Pyk2 and a receptor PTK, Epidermal Growth Factor Receptor (EGFR). Activation of these PTKs is dependent on the GPCR and appears to be cell-type specific. For instance, the Src-family PTK Lyn is essential for m1, but not m2 muscarinic acetylcholine receptor-induced MAPK activation, while the PTK Syk is necessary for both (4). Src is activated in response to LPA and mediates MAPK activation in COS-7 and PC12 cells (5,6), but not in fibroblasts, since MAPK is still activated in $Src^{-/-}$ fibroblasts in response to LPA (7). In PC12 cells, Src cooperates with Pyk2 in linking GPCRs, including receptors for bradykinin and LPA, to MAPK activation (6). The EGFR has been found to link GPCR-activation to MAPK activation, since LPA-, ET-1- and thrombin-induced MAPK activation is blocked by an EGFR selective PTK inhibitor and by overexpression of dominant negative EGFR (8). EGFR tyrosine phosphorylation (transactivation) may be involved in GPCR-induced MAPK activation (8). However, other reports indicate that EGFR tyrosine phosphorylation is not induced in response to GPCR activation (6,7). Therefore, the mechanism by which the EGFR is involved in GPCR-mediated MAPK activation remains to be determined definitively.

Here we report that LPA and ET-1 did not induce MAPK activation in SK-N-MC cells. However, SK-N-MC cells were responsive to these ligands, in that LPA and ET-1 induced a rapid, transient increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). Transient transfection of the EGFR into SK-N-MC cells potentiated LPA- and ET-1-induced MAPK activation, but LPA and ET-1 did not enhance basal level EGFR tyrosine phosphorylation. Our results demonstrate that introduction of the EGFR is sufficient to potenti-

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ate GPCR-induced MAPK activation in these cells, bolstering the idea that the EGFR is involved in GPCR-induced MAPK activation.

EXPERIMENTAL PROCEDURES

Cells, plasmids, and materials. SK-N-MC neuroepithelioma cells and COS-7 cells were cultured in a 1:1 mixture of DMEM and Ham's F12 (DF) medium supplemented with 10% and 7.5% foetal calf serum, respectively. The SV40-driven expression vector for the human EGFR, pSV2HERc, has been described before (9). The cytomegalovirus (CMV) promoter driven expression vector for haemagglutinin (HA)-epitope-tagged p42 MAPK (10) was a kind gift of Mike Weber. LPA and ET-1 were bought from Sigma (St Louis, MO, USA), recombinant bovine basic Fibroblast Growth Factor (bFGF) from Boehringer Mannheim (Germany), recombinant Platelet Derived Growth Factor (PDGF) from PreproTech Inc. (NJ, USA) and purified EGF from Biomedical Technologies Inc. (MA, USA).

Transient transfections and MAPK activity assays. Transient transfections by calciumphosphate precipitation were done exactly as described before (11). Following stimulation with the indicated stimuli for 5 min, the cells were lysed in cell lysis buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 10 u/ml aprotinin, 1 μM pMSF, 200 μM sodium orthovanadate). Immunoprecipitation was done by incubation with anti HA-tag MAb (12CA5) and protein A-sepharose (Pharmacia, Uppsalala, Sweden) for 3 h at 4°C. The beads were washed extensively and resuspended in kinase buffer (40 mM Tris pH 8.0, 20 mM $MgCl_2$, 2 mM $MnCl_2$, 10 μM ATP) containing 1 μCi γ-[^{32}P]-ATP and 10 μg myelin basic protein (MBP) as a substrate per sample and incubated for 30 min at 30°C, followed by addition of Laemmli sample buffer, boiling and loading on 15% SDS-polyacrylamide gels. The kinase assays were quantified using a Phosphorlmager (Molecular Dynamics) and visualized by autoradiography.

$[Ca^{2+}]_i$ measurements. SK-N-MC cells, attached to a glass substrate, were incubated in Hepes buffered saline (HBS: 10 mM HEPES, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, pH 7.3 at 33°C) with 10 μM Indo-1-AM (Molecular Probes, OR, USA) for 45 min. Coverslips were mounted in a quartz cuvette and measurements were done using a Perkin Elmer model LS50B fluorescence spectrometer at 33°C (excitation 355 nm [slit 5 nm] and 405 nm emission [slit 10 nm]). Calibration of the internal calcium was determined using 5 μg/ml ionomycin. The maximal and minimal fluorescence was determined by addition of 4 mM $CaCl_2$, and 6 mM $MnCl_2$, respectively in the presence of ionomycin. Absolute values were calculated according to Grynkiewitcz et al. (12).

Immunoprecipitation and immunoblotting. The EGFR was immunoprecipitated using MAb 108.1 (13). Immunoblotting was done exactly as described before (11), using anti-P.Tyr MAb PY-20 (Transduction Laboratories, KY, USA) or polyclonal rabbit anti-EGFR antibody 281-7 (14) (kind gift of Bas Defize). Detection of the immunoreactive bands using horse radish peroxidase-conjugated secondary antibodies was done by enhanced chemiluminescence (ECL).

RESULTS

Lack of MAPK activation in response to LPA in SK-N-MC cells. We investigated MAPK activation in response to a panel of stimuli in SK-N-MC neuroepithelioma cells. The cells were transiently transfected with epitope-tagged MAPK (HA-MAPK), stimulated for 5 min with LPA, FGF or EGF, and MAPK activity was determined by a kinase assay, using myelin basic protein (MBP) as a substrate. Whereas FGF induced a 15-

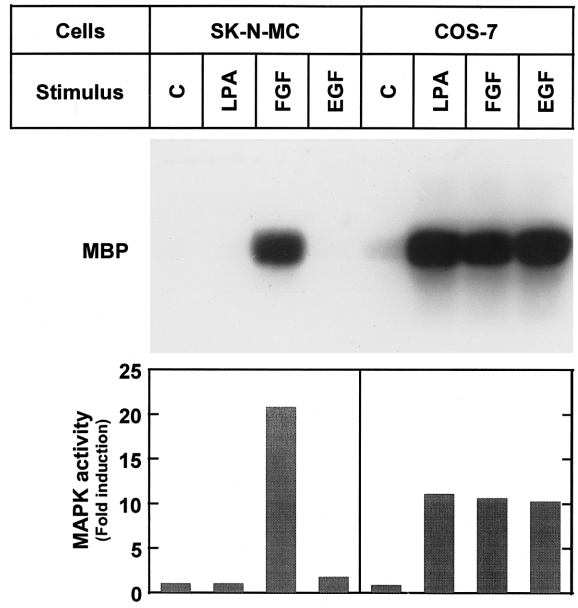

FIG. 1. LPA did not induce MAPK activation in SK-N-MC cells. SK-N-MC cells and, as a control, COS-7 cells were transiently transfected with HA-tagged MAPK, serum-starved overnight, and treated for 5 min with LPA (1 μM), FGF (10 ng/ml) or EGF (50 ng/ml), or they were left untreated (control, C). HA-tagged MAPK was immunoprecipitated and an *in vitro* kinase assay using γ-[^{32}P]-ATP and MBP as a substrate was performed. Autoradiographs of the 15% SDS-polyacrylamide gel of a representative experiment are shown in the upper panel. Direct quantification of the radioactivity incorporated in MBP was done using a Phosphorlmager, and the results are depicted in the lower panel as fold induction relative to the control.

to 25-fold stimulation of MAPK activity, LPA nor EGF elicited detectable MAPK activation in these cells (Figure 1). SK-N-MC cells are not responsive to EGF, since these cells do not express endogenous EGFRs (15, 16). In parallel to the SK-N-MC cells we determined MAPK activation in response to the same panel of stimuli in COS-7 cells. All three stimuli induced strong MAPK activation (8- to 14-fold), depending on the stimulus (Figure 1). These results demonstrate that SK-N-MC cells are not responsive to LPA with respect to MAPK activation.

LPA- and ET-1-induced Ca^{2+} mobilization in SK-N-MC cells. Most cell types are responsive to LPA, indicating that most cell types express LPA receptors. It is well established that LPA elicits a rapid, transient rise in $[Ca^{2+}]_i$ in LPA-responsive cells, including fibroblasts (17). Since LPA did not induce MAPK activation in SK-N-MC cells (Figure 1), we investigated the effects of LPA on $[Ca^{2+}]_i$, in order to establish whether SK-N-MC cells expressed LPA receptors. LPA-treatment led to a rapid, transient increase in $[Ca^{2+}]_i$ of approximately 190 nM (Figure 2A,C), which is in the same range as the LPA-induced rise in $[Ca^{2+}]_i$, reported in fibroblasts (18). ET-1, a ligand for another GPCR, also induced a rise in $[Ca^{2+}]_i$ of approximately

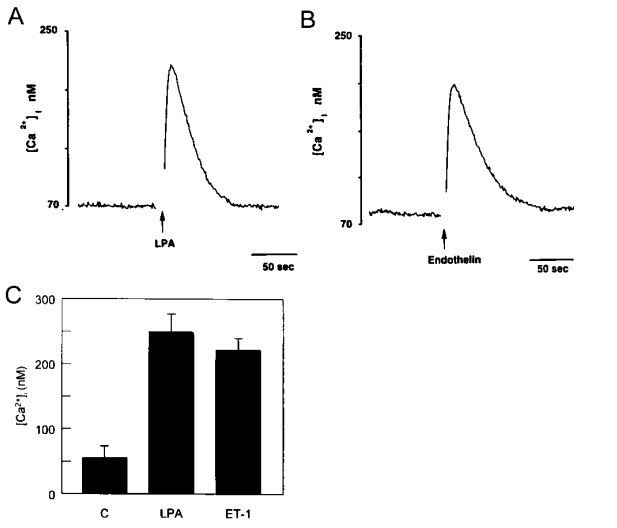

FIG. 2. LPA and ET-1 induced a rapid, transient rise in $[Ca^{2+}]_i$ in SK-N-MC cells. INDO.1.AM-loaded SK-N-MC cells were challenged with (A) LPA (1 μM) or (B) ET-1 (100 nM), while constantly monitoring fluorescence. Representative traces are depicted. (C) Quantification of LPA- and ET-1-induced changes in $[Ca^{2+}]_i$. The average of 4 independent experiments is depicted and the standard deviation is indicated.

160 nM (Figure 2B,C). EGF did not affect $[Ca^{2+}]_i$ in SK-N-MC cells, since these cells do not express EGFRs (data not shown). These results clearly demonstrate that SK-N-MC cells are responsive to LPA and ET-1. Therefore, we conclude that SK-N-MC cells express receptors for LPA and ET-1.

Potentiation of GPCR-induced MAPK activation by overexpression of the EGFR. LPA did not induce MAPK activation in SK-N-MC cells (Figure 1), even though these cells expressed functional LPA receptors (Figure 2). Daub et al. (8) have demonstrated that the EGFR may link GPCRs to MAPK activation. The fact that SK-N-MC cells do not express endogenous EGFRs (15,16) prompted us to test the hypothesis that the lack of endogenous EGFRs is responsible for the fact that GPCR activation did not induce MAPK activation in these cells. To this end we co-transfected the EGFR and epitope-tagged MAPK into SK-N-MC cells and determined MAPK activity, following stimulation with a panel of stimuli. LPA induced a 5- to 12-fold increase in MAPK activity in SK-N-MC cells, co-transfected with EGFR, but not in mock-transfected cells. Likewise, ET-1 did not induce MAPK activation in mock-transfected cells, but elicited a 14- to 23-fold induction of MAPK-activity in EGFR-transfected cells (Figure 3). Relative MAPK activation varied from experiment to experiment, presumably due to subtle differences in experimental conditions. However, LPA and ET-1 always induced significant MAPK activation in EGFR-transfected cells, but never in mock-transfected cells. The response to PDGF (13- to 45-fold MAPK activation) was not dependent on co-transfection of the EGFR (Figure 3). The EGFR is functional in transiently transfected SK-N-MC cells, since EGF itself also induced MAPK-activation (32- to 62-fold) in EGFR-transfected, but not mock-transfected cells. These results demonstrate that overexpression of the EGFR potentiated GPCR-induced MAPK activation in SK-N-MC cells.

LPA and ET-1 did not induce EGFR transactivation in SK-N-MC cells. Whether GPCR activation induces EGFR tyrosine phosphorylation (transactivation) is currently under debate (6–8, 19). In order to establish whether LPA and ET-1 induced EGFR transactivation in SK-N-MC cells, we determined EGFR tyrosine phosphorylation by immunoblotting. Transient transfection of the EGFR in SK-N-MC cells resulted in low basal level tyrosine phosphorylation of the EGFR. LPA nor ET-1 induced enhanced EGFR tyrosine phosphorylation, while EGF elicited a robust increase in EGFR tyrosine phosphorylation (Figure 4). These results demonstrate that transiently transfected EGFR is functional and suggest that LPA- and ET-1-induced MAPK activation in transiently transfected SK-N-MC cells is not due to EGFR transactivation.

DISCUSSION

Activation of GPCRs induces activation of MAPK via an intermediary PTK. Identification of this intermedi-

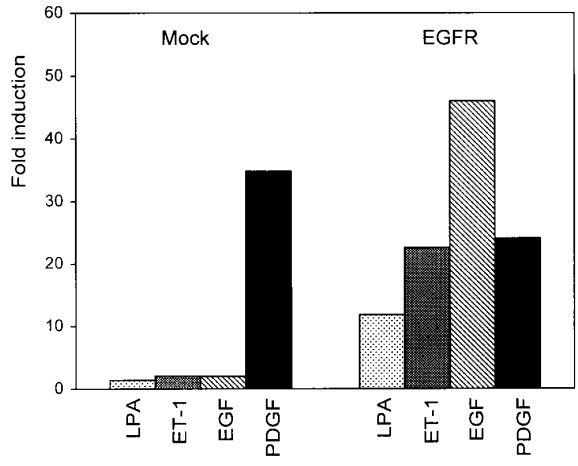

FIG. 3. The EGFR potentiated MAPK activation in response to GPCR activation. SK-N-MC cells were transiently co-transfected with HA-epitope-tagged MAPK and expression vector (Mock) or pSV2HERc, an expression vector for the human EGFR. The cells were serum-starved overnight and left untreated, or they were treated with LPA (1 μM), ET-1 (100 nM), EGF (50 ng/ml), or PDGF (50 ng/ml) for 5 min. Subsequently, the cells were lysed, epitope-tagged MAPK was immunoprecipitated, and a kinase assay was performed, using MBP as a substrate. Equal HA-MAPK expression was monitored by immunoblotting (data not shown) and MAPK-activity was determined using a Phosphorlmager. MAPK activation is depicted as fold induction, relative to the mock-transfected or EGFR-transfected unstimulated controls, respectively.

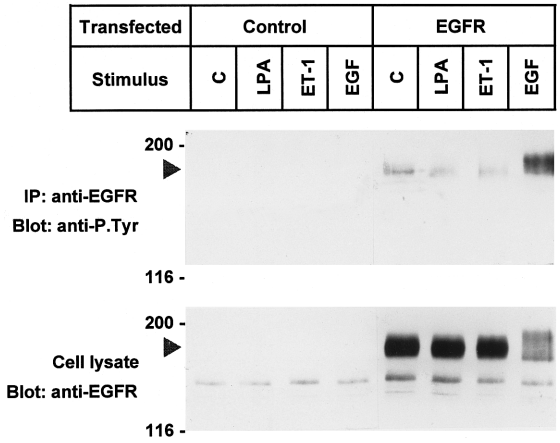

FIG. 4. Lack of EGFR transactivation in response to LPA and ET-1 in SK-N-MC cells. SK-N-MC cells were transiently transfected with an expression vector for the human EGFR, pSV2HERc. The cells were serum-starved overnight and left untreated (C), or they were treated with LPA (1 μM), ET-1 (100 nM), or EGF (50 ng/ml) for 5 min. Subsequently the cells were lysed, the EGFR was immunoprecipitated, and either the immunoprecipitates (upper panel) or total cell lysates (lower panel) were loaded on 7.5% SDS-PAGE gels. The material on the gels was transferred to blots and the blots were probed with anti-P.Tyr MAb (PY-20) (upper panel) or anti-EGFR polyclonal antibody (281-7) (lower panel) and developed using enhanced chemiluminescence (ECL). Immunoblots are depicted with the molecular weights (in kDa) of marker proteins that were co-electrophoresed with the samples on the left. The position of the EGFR is indicated.

ary PTK has been the subject of several recent studies and at least five PTKs have been found that may mediate GPCR-induced MAPK activation. Here we provide evidence supporting the model that the EGFR, one of the candidate PTKs, is involved in MAPK activation in response to LPA and ET-1, since transfection of the EGFR in SK-NM-C cells, that lack endogenous EGFR, potentiates MAPK activation by these two GPCR agonists.

The mechanism by which GPCR activation leads to MAPK activation remains to be determined definitively. LPA is the prototypic GPCR ligand that induces MAPK activation. The LPA receptor mediates its effects through at least three distinct heterotrimeric G proteins, G_q, $G_{12/13}$ and G_i (2). $G_{12/13}$ may link the LPA receptor to the small GTP-binding protein Rho, eventually leading to cytoskeletal remodelling and cell shape changes (20,21). G_q links to phospholipase Cβ, leading to the production of diacylglycerol and inositol tris-phosphate (IP_3), resulting in activation of Protein Kinase C (PKC) and Ca^{2+}-mobilization, respectively (22). It has been speculated that G_q-mediated activation of PKC may lead to MAPK activation in a Ras-independent manner (1,23). However, G_q-mediated MAPK activation was only partially inhibited following downregulation of PKC, indicating that G_q-mediated MAPK activation is largely PKC-independent (24). Moreover, pertussis toxin completely abolished LPA-induced MAPK activation in Rat-1 fibroblasts (25), indicating that LPA-induced MAPK activation is mediated solely by pertussis toxin sensitive G_i and not by G_q. Here we demonstrate that LPA elicited a rise in $[Ca^{2+}]_i$ in SK-N-MC cells, which is presumably mediated by G_q. However, LPA did not induce MAPK activation in these cells, suggesting that G_q-activated pathways are not involved in MAPK activation in SK-N-MC cells.

G_i-mediated MAPK activation is most likely mediated by the $G_i\beta\gamma$ subunits, rather than the α_i subunit (5,26,27), and involves an intermediary PTK, since genistein and staurosporine, non-specific inhibitors of PTK activity, abolished activation of the Ras/MAPK pathway (3,7,25,28). Several candidate PTKs have been identified that may mediate MAPK activation in response to GPCR agonists (4–6,8). The EGFR may link GPCRs to MAPK activation in Rat-1 fibroblasts, since LPA-, ET-1- and thrombin-induced MAPK activation was blocked by overexpression of dominant negative EGFR, and by an EGFR selective PTK-inhibitor (8). More detailed analysis of the involvement of the EGFR in GPCR-mediated MAPK activation indicated that Src is involved as well, since the Src-specific inhibitor, PP1, severely inhibited LPA- and EGF-induced MAPK activation (19). However, the involvement of Src in LPA-induced MAPK activation is complex, since MAPK activation was still observed in $Src^{-/-}$ cells (7). We demonstrate here that SK-N-MC cells are not responsive to LPA and ET-1 with respect to MAPK activation. Src immunoprecipitation/ kinase assays indicated that SK-N-MC cells express Src at levels that are comparable to fibroblasts (data not shown), suggesting that Src is not sufficient for LPA- and ET-1-induced MAPK activation. Overexpression of Src by itself induced MAPK activation which was not enhanced further by LPA or ET-1 (data not shown). Therefore, we cannot exclude the possibility that Src is involved in GPCR-induced MAPK activation via the EGFR in SK-N-MC cells.

Transient transfection of the EGFR in SK-N-MC cells potentiated LPA- and ET-1-induced MAPK activation. SK-N-MC cells do not express endogenous EGFR. However, these cells express functional receptor PTKs, including FGFR and PDGFR (15, 16 and Figures 1 and 3). Apparently, endogenous FGFR and PDGFR do not mediate MAPK activation in response to GPCR agonists. It is noteworthy that dominant negative EGFR and specific EGFR inhibitors inhibited GPCR-induced MAPK activation (8,19), suggesting that the EGFR specifically mediates GPCR-induced MAPK activity.

Transient transfection of the EGFR in SK-N-MC cells led to low basal levels of EGFR autophosphorylation as determined by anti-P.Tyr immunoblotting, conceivably due to EGFR overexpression at relatively high levels (Figure 4). LPA and ET-1 did not induce en-

hanced tyrosine phosphorylation of the EGFR, while EGF did, suggesting that establishment of GPCR-induced MAPK activation by transient transfection of the EGFR is not due to transactivation of the EGFR, as described in previous reports (8,19). Our data are consistent with several reports that fail to detect EGFR transactivation in response to GPCR activation (6,7). The mechanism by which the EGFR is involved in GPCR-induced MAPK activation in SK-N-MC cells remains to be determined. It is noteworthy that PDGF-induced MAPK activation, which is independent of EGFR-signalling, is similar in mock-transfected and EGFR transfected cells (Figure 3), indicating that transient transfection of the EGFR by itself did not enhance MAPK activation in general. Transfection of the EGFR into SK-N-MC cells led to low basal level tyrosine phosphorylation of the EGFR itself (Fig. 4), but did not induce major increases in tyrosine phosphorylation of cellular proteins (data not shown). LPA and ET-1 did not enhance EGFR tyrosine phosphorylation and we did not detect significant changes in tyrosine phosphorylation of cellular proteins, as determined by anti-P.Tyr immunoblotting of total cell lysates (data not shown). However, we cannot exclude the possibility that subtle changes in tyrosine phosphorylation are induced by LPA and ET-1. Transient transfection of the EGFR clearly potentiated GPCR-induced MAPK activation in SK-N-MC cells. Since transactivation apparently is not involved in GPCR-induced MAPK activation in SK-N-MC cells, we hypothesize that the basal level EGFR tyrosine phosphorylation that we observed may be sufficient to potentiate GPCR-induced MAPK activation, for instance by recruiting adaptor proteins and/or other signalling proteins to the membrane. Alternatively, basal level EGFR PTK activity may lead to subtle changes in P.Tyr-content of key substrates, thereby potentiating GPCR-induced MAPK activation.

Here we demonstrate that transient transfection of the EGFR potentiated GPCR-induced MAPK activation in SK-N-MC cells that are normally not responsive to GPCR-activation with respect to MAPK activation, bolstering the idea that the EGFR is involved in linking GPCRs to MAPK activation. Since transient transfection of the EGFR is sufficient to potentiate GPCR-induced MAPK activation in SK-NM-C cells, these cells may be a useful tool to dissect the mechanism of EGFR-mediated GPCR-induced MAPK activation.

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