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Galardin (GM 6001), a broad-spectrum matrix metalloproteinase inhibitor, blocks bombesin- and LPA-induced EGF receptor transactivation and DNA synthesis in Rat-1 cells☆

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Abstract

Matrix metalloproteinases (MMPs) have been implicated in the transactivation of the epidermal growth factor receptor (EGFR) induced by G-protein coupled receptor (GPCR) agonists. Although EGFR phosphorylation and downstream signaling have been shown to be dependent on MMP activity in many systems, a role for MMPs in GPCR-induced DNA synthesis has not been studied in any detail. In this study we utilized the broad-spectrum matrix metalloproteinase inhibitor, galardin (Ilomastat, GM 6001), to study the mechanism of bombesin- or LPA-induced EGFR transactivation and the role of MMPs in early and late response mitogenic signaling in Rat-1 cells stably transfected with the bombesin/GRP receptor (BoR-15 cells). Addition of galardin to cells stimulated with bombesin or LPA specifically inhibited total EGFR phosphorylation, as well as site-specific phosphorylation of tyrosine 845, a putative Src phosphorylation site, and tyrosine 1068, a typical autophosphorylation site. Galardin treatment also inhibited extracellular signal-regulated kinase (ERK) activation induced by bombesin or LPA, but not by EGF. In addition, galardin inhibited bombesin- or LPA-induced DNA synthesis in a dose dependent manner, when stimulated by increasing concentrations of bombesin, and when added after bombesin stimulation. Furthermore, addition of galardin post-bombesin stimulation indicated that by 3 h sufficient accumulation of EGFR ligands had occurred to continue to induce transactivation despite an inhibition of MMP activity. Taken together, our results suggest that MMPs act as early as 5 min, and up to around 3 h, to mediate GPCR-induced EGFR transactivation, ERK activation, and stimulation of DNA synthesis.

Keywords: Ilomastat; Lysophosphatidic acid; Cell cycle activation; G-protein coupled receptors; MMP; Epidermal growth factor receptor

Introduction

Neuropeptides and bioactive lipids stimulate DNA synthesis and cell proliferation in cultured cells and are implicated as growth factors in a variety of fundamental processes including development, inflammation, tissue regeneration, and neoplastic transformation. Binding of these agonists to their cognate G-protein coupled receptors[1] (GPCRs) promote the $G\alpha_q$-mediated activation of β isoforms of phospholipase C [1–3] to produce the second messengers inositol 1,4,5-trisphosphate and diacylglycerol [4–6]. These receptors also interact with members of the $G\alpha_{12}$ family leading to an increase in tyrosine phosphory-

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[1] Abbreviations used: AG, AG 1478; bom, bombesin; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular signal regulated kinase; FAK, focal adhesion kinase; FBS, fetal bovine serum; gal, galardin; GPCR, G-protein coupled receptor; GRP, gastrin releasing peptide; HB-EGF, heparin bound epidermal growth factor like growth factor; LPA, lysophosphatidic acid; mAb, monoclonal antibody; mapk, mitogen activated protein kinase; MMP, matrix metalloproteinase; PBS, phosphate buffered saline; PKC, protein kinase C; proHB-EGF, proheparin bound epidermal growth factor like growth factor; RIPA, radioimmunoprecipitation; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel eletrophoresis; TBS, Tris buffered saline; TCA, trichloroacetic acid; TGF-α, transforming growth factor-α; TNF-α, tumor necrosis factor-α; Tyr(P), phosphotyrosine

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lation of focal adhesion proteins. Recently, rapid tyrosine phosphorylation of the epidermal growth factor receptor (EGFR), a process termed EGFR transactivation, was also found to be induced by GPCR agonists [7,8]. Subsequently, these agonists induce striking activation of serine phosphorylation cascades including extracellular signal-regulated kinases 1 and 2 (ERK-1/2) [9–12] leading to increased expression of immediate early response genes, stimulation of cell cycle progression into S phase, and cell proliferation [6,13–16].

The mechanism by which GPCR agonists induce EGFR transactivation is not well understood. Two central mechanisms have been proposed, namely, phosphorylation of the EGFR by the nonreceptor tyrosine kinase Src [7,17–20], and matrix metalloproteinase (MMP)-mediated release of EGFR ligands [20–28]. For example, Src has been shown to directly phosphorylate the EGFR at Tyr-845 [29–33], a site important for EGF-induced mitogenesis. More recently, MMP-dependent release of the EGFR ligands HB-EGF and TGF-α, induced by GPCR agonists including bombesin and LPA, has been documented in an increasing number of systems [20–28]. A possible interplay between Src and MMPs in mediating GPCR-induced EGFR transactivation has been relatively unexplored, as has the contribution of MMPs in DNA synthesis.

The broad-spectrum matrix metalloprotease inhibitor galardin (GM 6001, Ilomastat) is a hydroxamic acid originally synthesized as an inhibitor of human skin collagenase [34], and has been shown to block MMP-1, -2, -3, and -9 [35,36]. In this study we used galardin to explore the mechanism by which bombesin and LPA induce EGFR transactivation in Rat-1 cells stably transfected with the bombesin/GRP receptor (BoR-15 cells). Our results show that galardin inhibited both overall EGFR tyrosine phosphorylation and phosphorylation of tyrosine 845 and 1068, suggesting that in Rat-1 cells, GPCR-induced EGFR transactivation is mediated by MMP-dependent release of EGFR ligands. At concentrations that abrogated EGFR phosphorylation, galardin also selectively inhibited ERK activation and attenuated the stimulation of DNA synthesis in response to bombesin or LPA. Furthermore, addition of galardin post-bombesin stimulation indicated that by 3 h sufficient accumulation of EGFR ligands had occurred to continue to induce transactivation despite an inhibition of MMP activity. These results suggest that MMPs act as early as 5 min, and up to around 3 h, to generate the release and accumulation of EGFR ligands that mediate bombesin-induced EGFR phosphorylation and ERK activation, and contributes to the stimulation of DNA synthesis.

Materials and methods

Cell culture

Stock cultures of Rat-1 cells transfected with the bombesin/GRP (BoR-15) receptor [6,37] were maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% fetal bovine serum (FBS) and 0.5 mg/ml G418 in a humidified atmosphere containing 10% carbon dioxide (CO_2) and 90% air. For experimental purposes, cells were plated on 100 mm or 35 mm dishes at 6×10^5 or 1×10^5 cells/dish, respectively, and grown in DMEM containing FBS and G418 for 4–6 days until confluent. The cultures were then switched to serum-free DMEM 24 h before use.

DNA synthesis measurements

Confluent cultures of BoR-15 cells were serum-starved in DMEM overnight, then washed twice with DMEM and incubated in DMEM-Waymouth's medium (1:1 vol/vol) and various additions as described in the legends for Figs. 4–6. After 20 h of incubation at 37°C, [^{3}H]thymidine (0.2 μCi/ml, 1μM) was added, and the cultures were incubated for a further 4 h at 37°C. Cells were then washed two times with phosphate buffered saline (PBS) and incubated in 5% trichloroacetic acid (TCA) at 4°C for 20 min to remove acid soluble radioactivity, washed with ethanol, and solubilized in 1 ml of 0.1 M sodium hydroxide (NaOH) and 1% sodium dodecyl sulfate (SDS). Acid insoluble radioactivity was determined by scintillation counting in 6 ml of Beckman Readysafe.

EGFR tyrosine phosphorylation assay

Confluent cultures of BoR-15 cells grown on 100 mm dishes were serum starved in DMEM overnight, then washed twice with serum-free DMEM and incubated for 1 h at 37°C with or without inhibitors, as indicated. Cells were then treated for 5 min with agonists. The stimulation was terminated on ice by aspirating the medium and lysing the cells in 1 ml ice-cold modified radioimmunoprecipitation (RIPA) buffer [50 mM Tris · HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM Pefabloc SC, 1 mg/ml each of aprotinin, leupeptin, and pepstatin, 1 mM sodium pervanadate (Na_3VO_4), and 1 mM sodium fluoride (NaF)]. The cell suspension was then mixed for 20 min at 4°C to lyse the cells, and cell lysates were clarified by centrifigation at 15,000 rpm for 10 min. For tyrosine phosphorylation of the EGFR, the supernatants were transferred to new microtubes and mixed with 10 μg/ml polyclonal α-EGFR at 4°C overnight. The immunocomplex was captured by adding 100 μl protein-A-agarose beads for 1 h, then agarose beads were collected by pulse centrifugation (0.5 min at 14,000 rpm). The beads were washed three times with ice-cold RIPA buffer and subsequently solubilized in 100 μl 2× sodium dodecyl sulfate-polyacrylamide gel eletrophoresis (SDS-PAGE) sample buffer. For phosphorylation of tyrosine 845 and 1068 on the EGFR, the supernatants were transferred to new microtubes and mixed with an equal volume of 2× SDS-PAGE sample buffer. In both cases, samples were then

boiled for 15 min, and proteins resolved by 8% SDS-PAGE, then transferred to Immobilon-P membranes. Tyrosine-phosphorylated EGFR was detected by immunoblotting using 4G10 anti-phosphotyrosine [anti-Tyr(P)] monoclonal antibody (mAb) and tyrosine 845 and 1068 phosphorylation were detected by immunoblotting with polyclonal anti-EGFR [pY845] and anti-EGFR [pY1068].

ERK activation assay

Confluent cultures of BoR-15 cells grown on 35 mm dishes were serum starved in DMEM overnight, then washed two times with DMEM and incubated for 1 h at 37°C with or without inhibitors, as indicated. The cultures were then treated with agonist and incubated for a further 5 min at 37°C. The stimulation was terminated on ice by aspirating the medium and solubilizing the cells with 150 μl of 2× SDS-PAGE sample buffer (200 mM Tris · HCl, pH 6.8, 1 mM EDTA, 6% SDS, 2 mM EDTA, 4% 2-mercaptoethanol, and 10% glycerol). Samples were boiled for 20 min, resolved by 10% SDS-PAGE, and transferred to Immobilon-P membranes. Activated forms of ERK-1 and ERK-2 were monitored using a specific anti-phospho-ERK-1/2 monoclonal antibody that recognizes only the activated forms phosphorylated on both Thr^{202} and Tyr^{204}.

Immunoblotting

After SDS-PAGE, proteins were transferred to Immobilon-P membranes. For detection of proteins, membranes were blocked for at least 1 h at room temperature using 5% nonfat dried milk in PBS (pH 7.2) and then incubated overnight with the desired antibodies diluted in Tris buffered saline (TBS) (pH 7.2) containing 0.1% Tween-20 and 3% nonfat dried milk. Primary antibodies bound to immunoreactive bands were visualized by enhanced chemiluminescence detection with horseradish peroxidase-conjugated anti-mouse or anti-rabbit.

Materials

Bombesin, EGF, insulin, LPA, and tyrphostin AG 1478 were obtained from Sigma (St. Louis, MO, USA). [^{3}H]thymidine was from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Protein A-agarose was from Boehringer Mannheim (Indianapolis, IN, USA). Galardin was purchased from Calbiochem (San Diego, CA, USA). The polyclonal anti-ERK-2 was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 4G10 anti-Tyr(P) mAb and anti-EGFR were from Upstate Biotechnology (Lake Placid, NY, USA). Anti-phospho-ERK-1/ERK-2 mAb was obtained from Cell Signaling (Beverly, MA, USA). Anti-EGFR [pY845] and anti-EGFR [pY1068] were purchased from Biosource (Camarillo, CA, USA). All other materials were of the highest grade available.

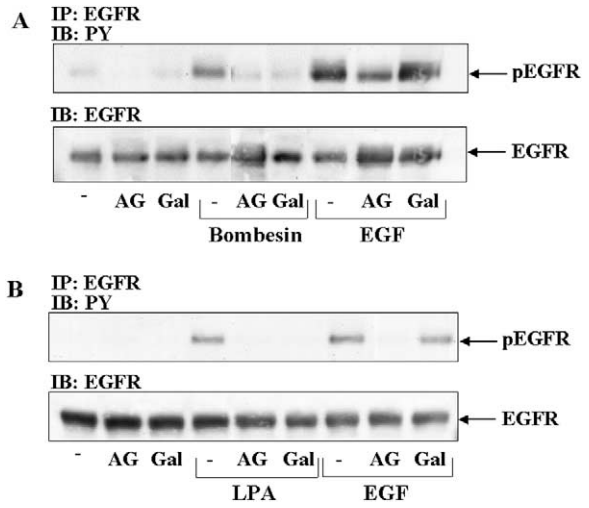

Fig. 1. Effect of galardin on total EGFR tyrosine phosphorylation induced by bombesin, LPA, and EGF. (A and B). Confluent and quiescent cells were stimulated for 5 min with 10 nM bombesin or 5 ng/ml EGF (A), or 10 μM LPA or 0.5 ng/ml EGF (B), after 1 h preincubation with 250 nM AG 1478 (AG) or 10 μM galardin (Gal), as indicated. The EGF receptor was immunoprecipitated using a polyclonal EGFR antibody, and resolved by SDS-PAGE. Proteins were transferred onto Immobilon-P membranes, then tyrosine phosphorylated EGFR was detected using 4G10 anti-Tyr(P) mAb. Membranes were then stripped and reprobed with anti-EGFR. The results presented here are representative of 2 independent experiments.

Results

Galardin inhibits bombesin- or LPA-induced total EGFR phosphorylation

To determine whether galardin (Ilomastat, GM 6001), a broad-spectrum matrix metalloproteinase inhibitor, could selectively prevent EGFR transactivation induced by stimulation with GPCR agonists, quiescent cultures of Rat-1 cells expressing the bombesin/GRP receptor (BoR-15 cells) were treated with or without 10 μM galardin for 1 h and then stimulated with bombesin, lysophosphatidic acid (LPA), or EGF for 5 min. Lysates of the cultures were immunoprecipitated with a specific anti-EGFR antibody and the immunoprecipitates were analyzed by Western blotting with α-Tyr(P) antibody. In accord with previous results [8], addition of bombesin to quiescent cultures of BoR-15 cells induced a striking increase in tyrosine phosphorylation of the EGFR, indicating that there was a robust cross-talk between this GPCR and the EGFR in this model system (Fig. 1A). Pretreatment of cells with galardin dramatically inhibited the increase in EGFR tyrosine phosphorylation induced by bombesin stimulation. The concentration of galardin used in these experiments, 10 μM, was the same as that shown to inhibit MMP-1, 2, and 3 [36].

To corroborate these results employing a GPCR endogenously expressed by Rat-1 cells, parallel experiments were performed using lysophosphatidic acid (LPA), a major bioactive lipid of serum [38]. LPA elicits a plethora of biological responses [39–41] via interaction with three recently

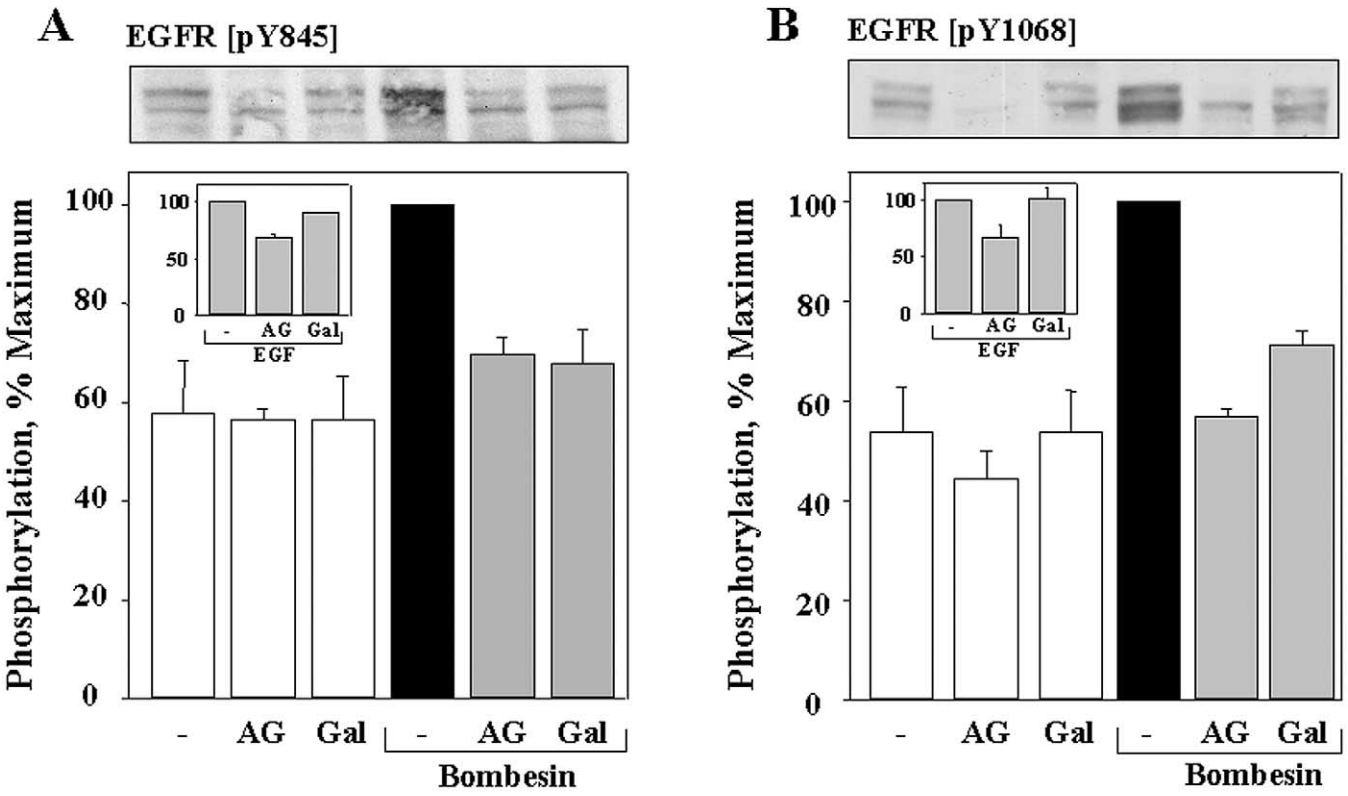

Fig. 2. Effect of galardin on phosphorylation of EGFR tyrosine 845 and tyrosine 1068 induced by bombesin. (A and B) Confluent and quiescent cells were stimulated for 5 min with 10 nM bombesin after 1 h preincubation with 250 nM AG 1478 or 10 μM galardin, as indicated. Cells were lysed in modified RIPA buffer, clarified, and supernatents were mixed 1:1 with 2 × SDS-PAGE sample buffer. Proteins were resolved by SDS-PAGE, transferred onto Immobilon-P membranes, then site-specific phosphorylation of the EGFR was detected using polyclonal anti-EGFR [pY845] (A) or anti-EGFR [pY1068] (B), as shown. The autoradiograms shown are representative of 3 independent experiments. The bars represent the quantification of the levels of EGFR Tyr-845 and Tyr-1068 phosphorylation analyzed by scanning densitometry. Results are expressed as a percentage of the maximum increase in phosphorylation and are the means ± S.E. of three independent experiments. *Insert, Effect of galardin on phosphorylation of EGFR Tyr-845 and Tyr-1068 induced by EGF.* Cells were stimulated for 5 min with 0.5 ng/ml EGF after 1 h preincubation with 250 nM AG 1478 or 10 μM galardin, as indicated.

identified heptahelical receptors [39,42,43], all of which couple to G_i and G_q, and two of which also couple to G_{12} [41,43,44]. As shown in Fig. 1B, pretreatment of cells with galardin also abrogated LPA-induced EGFR phosphorylation. The inhibition of bombesin- or LPA-induced EGFR phosphorylation by galardin is consistent with a role for MMP activity in GPCR-induced EGFR transactivation.

To verify that galardin effects GPCR-induced EGFR transactivation rather than interfering with direct EGF-induced signaling, Rat-1 cells were stimulated with two different concentrations of EGF, 5 ng/ml (Fig. 1A) or 0.5 ng/ml (Fig. 1B), after galardin pretreatment. The lower concentration of EGF induced a level of EGFR phosphorylation equivalent to that induced by LPA. Figs. 1A and B show that treatment with galardin did not effect EGFR phosphorylation induced by either 5 or 0.5 ng/ml of EGF, respectively, demonstrating that galardin specifically inhibits EGFR phosphorylation induced by stimulation of cells with GPCR agonists, but not by stimulation with an exogenously added EGFR ligand. In contrast, pretreatment of cells with AG 1478, a well established inhibitor of EGFR tyrosine kinase activity [45], blocked bombesin-, LPA-, and EGF-induced phosphorylation (Figs. 1A and B). The inhibition of EGF-induced EGFR phosphorylation by AG 1478, but not by galardin, suggests that galardin exerts its effects on GPCR-induced signaling at a level upstream of the activation of the EGFR.

Galardin inhibits bombesin- or LPA-induced EGFR phosphorylation at Tyr-845 and Tyr-1068

Subsequently, we examined the effect of galardin on the phosphorylation of specific EGFR sites: Tyr-845, a direct Src phosphorylation site that plays a critical role in EGF-induced mitogenesis [29], and Tyr-1068, a typical autophosphorylation site [46,47]. BoR-15 cells were treated with or without 250 nM AG 1478 or 10 μM galardin for 1 h, and then stimulated with 10 nM bombesin for 5 min. Cells were lysed in modified RIPA buffer and proteins were separated on a SDS-PAGE gel. As shown in Figs. 2A and B, bombesin induced a marked increase in the phosphorylation of both Tyr-845 and Tyr-1068 on the EGFR. Pretreatment of cells with galardin completely inhibited the increase in the phosphorylation of both residues induced by bombesin,

suggesting that the phosphorylation of both Tyr-845 and Tyr-1068 on the EGFR are dependent on MMP activity. In contrast, galardin did not inhibit EGF-induced EGFR Tyr-845 and Tyr-1068 phosphorylation (Fig. 2 inserts), indicating that MMP-mediated phosphorylation of these sites is specific to GPCR signaling, and hence occurs upstream of the EGFR. Taken together, the results presented in Figs. 1 and 2 indicate that galardin is a potent and selective inhibitor of GPCR-induced EGFR transactivation in BoR-15 cells.

Galardin inhibits bombesin- or LPA-induced ERK activation

Previously, we demonstrated that activation of the G_q-coupled bombesin receptor induces Ras/Raf/MEK/ERK activation in BoR-15 cells [6,37] through an EGFR-dependent pathway [8]. Other laboratories demonstrated that LPA induces ERK activation through G_i/Ras [38] in an EGFR-dependent manner [48] in Rat-1 cells. In order to explore whether galardin interferes with bombesin- or LPA-induced ERK activation, quiescent BoR-15 cells were treated with or without this compound for 1 h, followed by stimulation with bombesin, LPA, or EGF for 5 min. Activated forms of ERK-1 and ERK-2 from whole cell lysate were monitored using a specific anti-phospho-monoclonal antibody that recognizes only the activated forms phosphorylated on both Thr^{202} and Tyr^{204}.

As shown in Figs. 3A and B, bombesin or LPA treatment of BoR-15 cells induced an increase in ERK phosphorylation. This was blocked by pretreatment of cells with galardin. The effectiveness of this agent to prevent GPCR-induced ERK activation was comparable to that produced by direct inhibition of the EGFR tyrosine kinase with AG 1478, which is shown for comparison. In contrast, EGF-induced ERK activation was inhibited by AG 1478, but not by cell exposure to galardin. These results substantiate the notion that galardin selectively inhibits EGFR transactivation and ERK activation induced by GPCR agonists.

Galardin selectively attenuates bombesin- or LPA-induced DNA synthesis

Having established that galardin is a potent and selective inhibitor of bombesin- or LPA-induced EGFR tyrosine phosphorylation and ERK activation, we next used this agent to determine whether matrix metalloproteinases are required for GPCR-induced late response mitogenic signaling, i.e., re-entry into the S phase of the cell cycle. In order to examine whether MMP activity is necessary for bombesin- or LPA-induced DNA synthesis, quiescent BoR-15 cells were stimulated with 10 nM bombesin or 10 μM LPA in the presence of increasing concentrations of galardin (2.5–80 μM). Analysis of [^{3}H]thymidine incorporation into cells showed that galardin inhibited bombesin-induced DNA synthesis in a dose-dependent manner (Fig. 4A). At

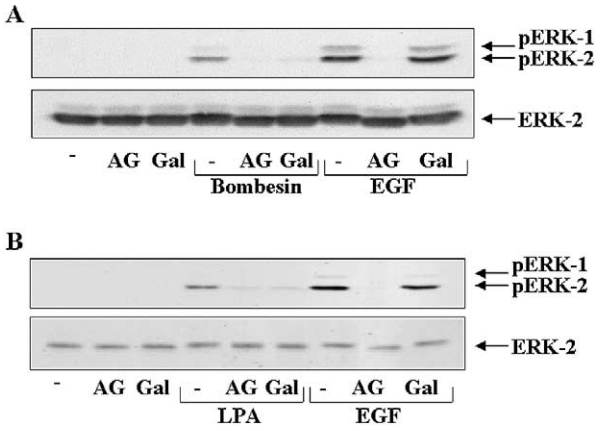

Fig. 3. Effect of galardin on ERK activation induced by bombesin, LPA, and EGF. Confluent and quiescent cells were stimulated for 5 min with 10 nM bombesin or 5 ng/ml EGF (A), or 10 μM LPA or 0.5 ng/ml EGF (B), after 1 h preincubation with 250 nM AG 1478 or 10 μM galardin, as indicated. Cells were lysed in 2 × SDS-PAGE sample buffer, and then proteins resolved by SDS-PAGE. Activated forms of ERK were monitored using a specific anti-phospho-ERK-1/ERK-2 mAb that recognizes only the activated form phosphorylated on Thr^{202} and Tyr^{204}. The results presented here are representative of 3 independent experiments.

~10 μM, a concentration that completely abrogated bombesin-induced EGFR transactivation (Figs. 1 and 2) and ERK pathway activation (Fig. 3), galardin produced 50% inhibition of DNA synthesis in response to bombesin. Galardin also inhibited LPA-induced DNA synthesis in BoR-15 cells in a dose dependent manner, with maximal inhibition occurring at 20 μM (Fig. 4B). The difference in the dose response curves between bombesin- and LPA-induced DNA synthesis could reflect a difference in the dependence of DNA synthesis on MMP-mediated EGFR transactivation, or a difference in the sensitivity of the MMPs activated by bombesin or LPA to galardin. In agreement with EGFR phosphorylation and ERK activation data, the inhibitory effect of galardin was specific to cells stimulated by GPCR agonists, since this compound did not prevent EGF-induced [^{3}H]thymidine incorporation in BoR-15 cells (Fig. 4A insert).

Bombesin induces DNA synthesis in BoR-15 cells in a dose-dependent manner [37]. In Fig. 5 we tested whether galardin could inhibit bombesin-induced [^{3}H]thymidine incorporation over a range of bombesin concentrations. Quiescent BoR-15 cells were incubated with increasing concentrations of bombesin, from 0.1 nM to 1000 nM, with or without a fixed concentration of galardin (10 μM). Galardin inhibited bombesin-induced [^{3}H]thymidine incorporation from 1 nM bombesin up to the highest concentration tested, 1 μM (Fig. 5). Even at the highest concentration of bombesin, 50% inhibition was achieved, consistent with the results obtained in Fig. 4A (50% inhibition at 10 μM galardin). Together, these results suggest that galardin-sensitive MMPs are necessary for maximal GPCR-induced DNA synthesis.

Time course of galardin inhibition of bombesin-induced DNA synthesis

Previously, we showed that EGFR tyrosine kinase activity is necessary in late G_1 for bombesin-induced DNA synthesis. For example, addition of AG 1478 up to 4 h after bombesin stimulation inhibited [^{3}H]thymidine incorporation to approximately the same degree as when AG 1478 was added together with bombesin in BoR-15 cells [8]. These results suggest that bombesin induces sustained EGFR transactivation, which is required for bombesin-induced DNA synthesis. However, how the EGFR is being continually activated is currently unknown. One possibility is the sustained activity of MMPs, which continuously generate EGFR ligands.

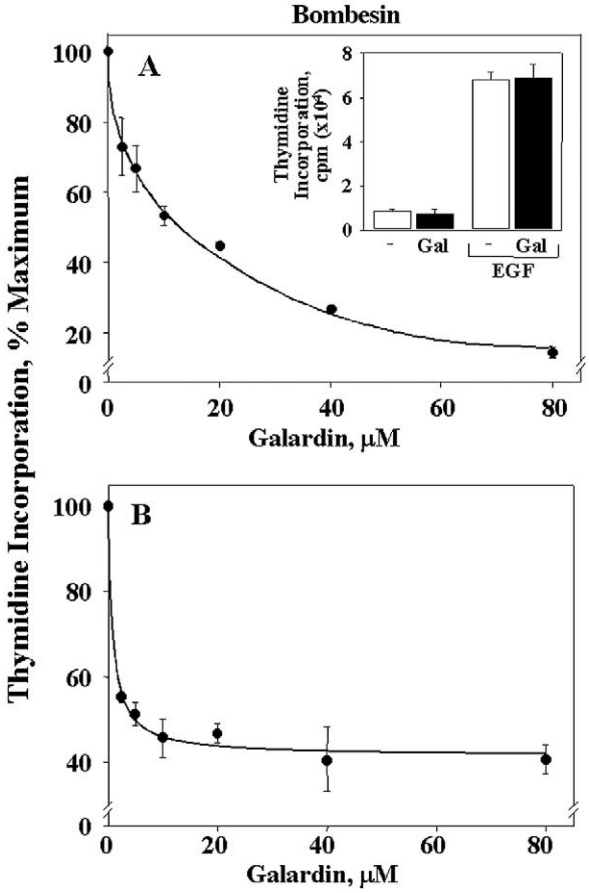

Fig. 4. Effect of increasing concentrations of galardin on bombesin- and LPA-induced DNA synthesis. (A and B). Confluent and quiescent cells were incubated in DMEM/Waymouth's medium containing 10 nM bombesin or 10 μM LPA, with or without increasing concentrations of galardin. Results are expressed as a percentage of incorporation induced by 10 nM bombesin or 10 μM LPA, and data are shown as a mean ± S.E. The results presented here are an average of 2 independent experiments. (A) The level of [^{3}H]thymidine incorporation into DNA was 2856 ± 588 cpm/dish in unstimulated cultures and 20422 ± 3458 cpm/dish in 10 nM bombesin treated cultures. *Insert, Effect of galardin on DNA synthesis induced by EGF*. Cells were incubated in DMEM/Waymouth's medium alone, or containing 10 μM galardin, 5 ng/ml EGF, or 5 ng/ml EGF with 10 μM galardin, as indicated. (B) The level of [^{3}H]thymidine incorporation into DNA was 2938 ± 167 cpm/dish in unstimulated cultures and 19295 ± 424 cpm/dish in 10 μM LPA treated cultures.

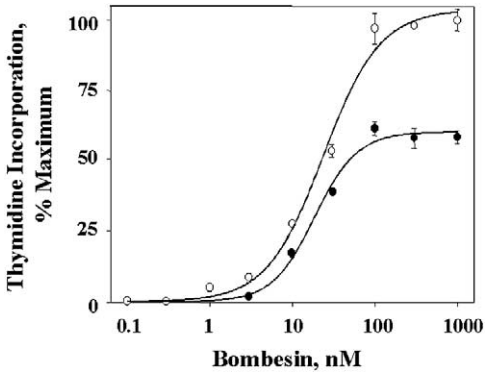

Fig. 5. Effect of galardin on DNA synthesis induced by increasing concentrations of bombesin. Confluent and quiescent cells were incubated in DMEM/Waymouth's medium containing increasing concentrations of bombesin with (●) or without (○) 10 μM galardin. Results are expressed as a percentage of incorporation induced by 1000 nM bombesin, and data are shown as a mean ± S.E. The results presented here are representative of 2 independent experiments. The level of [^{3}H]thymidine incorporation into DNA was 2926 ± 80 cpm/dish in unstimulated cultures and 30026 ± 1480 cpm/dish into bombesin treated cultures (100%).

To test the possibility that sustained MMP activity is required for GPCR-induced DNA synthesis, cultures of BoR-15 cells stimulated with bombesin received galardin at various times (−1 to 3 h, 6 h, and 14 h) after the addition of bombesin. In all cases, the cultures were labeled with [^{3}H]thymidine from 20–24 h, after which [^{3}H]thymidine incorporation into acid-insoluble pools was determined. If a quick burst, lasting only minutes, of MMP activity were sufficient to generate EGFR agonists for bombesin-induced DNA synthesis, then addition of galardin post-bombesin stimulation (i.e., starting from 1 h after) would have no effect on DNA synthesis. Conversely, if longer periods of MMP activity, lasting for hours, were required for DNA synthesis, then addition of galardin after bombesin treatment would give a similar degree of inhibition as if galardin were added 1 h before or simultaneously, with bombesin. Eventually, however, MMP activity would cease or sufficient EGFR agonist would accumulate, at which point adding galardin would no longer inhibit DNA synthesis. In agreement with the latter model, Fig. 6A shows that addition of galardin at times 1 h to 3 h after bombesin stimulation inhibited DNA synthesis by ~40%, i.e., to the same degree achieved when galardin was added to cells simultaneously with or 1 h before bombesin. Consistent with our previous findings with EGFR kinase inhibitors [8], these results suggest that prolonged MMP activity is necessary for bombesin-induced DNA synthesis.

By 5 h post-bombesin stimulation, [^{3}H]thymidine incorporation began to escape galardin inhibition, and by 14 h, incorporation into galardin-treated cells was similar to control, or non-treated cells (Fig. 6A). As a first step to explore the mechanism underlying the decrease in galardin sensi-

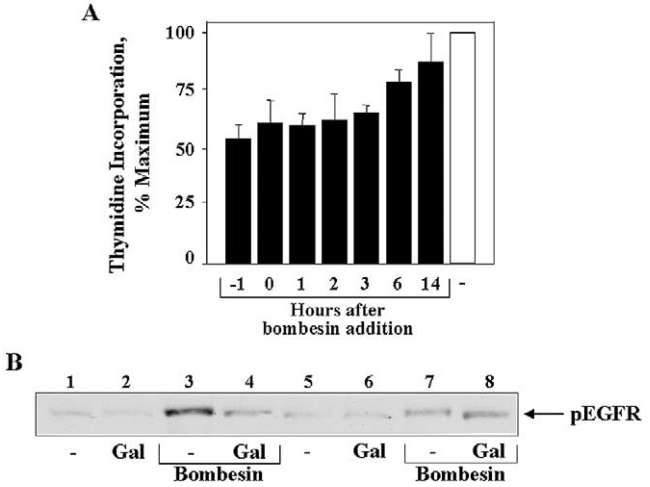

Fig. 6. Effect of the late addition of galardin on bombesin-induced DNA synthesis and total EGFR phosphorylation. (A) Confluent and quiescent cells were incubated in DMEM/Waymouth's medium containing 10 nM bombesin. 10 μM galardin was added to cells at 0, 1, 2, 3, 6, and 14 h after bombesin stimulation, as indicated (■). Results are expressed as a percentage of the incorporation induced by 10 nM bombesin (□), and data are shown as a mean ± S.E. The results presented here are representative of 3 independent experiments. The level of [^{3}H]thymidine incorporation into DNA was 1813 ± 727 cpm/dish in unstimulated cultures and 20199 ± 5965 cpm/dish into bombesin treated cultures (100%). (B) Confluent and quiescent cells were left untreated for 1 h (lane 1) or 5 hours (lane 5), treated with 10 μM galardin for 1 h (lane 2) or 5 h (lane 6), or treated with 10 nM bombesin for 5 min (lane 3) or 5 h (lane 7). Cells in lane 4 were pretreated with galardin for 1 h, then stimulated with bombesin for 5 min. Cells in lane 8 were stimulated with bombesin for 3 h, followed by addition of galardin for 2 h. The EGF receptor was immunoprecipitated using a polyclonal EGFR antibody, and resolved by SDS-PAGE. Proteins were transferred onto Immobilon-P membranes, then tyrosine phosphorylated EGFR was detected using 4G10 anti-Tyr(P) mAb. Membranes were then stripped and reprobed with anti-EGFR. The results presented here are representative of 3 independent experiments.

tivity, we stimulated BoR-15 cells with bombesin for 3 h and then added galardin for 2 h, and total EGFR tyrosine phosphorylation was compared to cells in which bombesin was added for a total of 5 h. Cells were also treated as in Fig. 1A in order to compare the level of inhibition of galardin when added before bombesin addition versus after bombesin addition. In agreement with the results presented in Fig. 1, bombesin induced a striking increase in EGFR phosphorylation, which was blocked by galardin when the inhibitor was added before bombesin stimulation (Fig. 6B, lanes 3 and 4). In cultures treated with bombesin for 5 h, EGFR phosphorylation was still apparent, albeit at a lower level than that seen in cultures treated with bombesin for 5 min (lanes 7). The salient feature of the results shown in Fig. 6 is that bombesin stimulation of the cells for 3 h, followed by galardin treatment for 2 h resulted in the same degree of EGFR phosphorylation as a 5 h bombesin treatment (Fig. 6B, compare lanes 8 and 7, respectively). The fact that galardin failed to inhibit EGFR phosphorylation when added 3 h after bombesin suggests that sufficient EGFR ligands accumulated to activate the EGFR despite suppression of MMP activity. Taken together, our results suggest that MMPs act as early as 5 min, and up to at least 3 h, to mediate bombesin-induced EGFR transactivation, ERK activation, and stimulation of DNA synthesis.

Discussion

Neuropeptides have been identified as potent cellular growth factors for normal cells and participate in autocrine/paracrine stimulation of tumor cell proliferation and migration. Recent studies on the signaling pathways activated by mitogenic neuropeptides through their cognate GPCRs revealed previously unsuspected connections and complexities, including the realization that these receptors not only stimulate the synthesis of conventional second messengers but also induce tyrosine phosphorylation cascades [49,50]. In particular, an increase in the tyrosine kinase activity of the EGFR has been identified as an important element in the complex signaling network that is stimulated by GPCR agonists in a variety of cell types. The mechanism by which GPCR agonists induce EGFR transactivation is not well understood.

Accumulated evidence indicates that Src kinases play crucial roles in the process of EGFR transactivation, leading to the phosphorylation of a specific residue in the EGFR, namely Tyr-845 [30,51]. Moreover, the recent identification of MMPs and transmembrane growth factor precursors as critical elements in GPCR-induced EGFR transactivation pathways has identified new components of receptor cross-talk of rapidly increasing complexity. For example, MMP-dependent cleavage of proHB-EGF was found to mediate GPCR-induced EGFR phosphorylation, adapter protein phosphorylation and complex formation, and ERK activation [21–23, 25–28]. Additionally, transforming growth factor-α (TGF-α) has recently been shown to mediate prostaglandin E_2- and carbachol-induced EGFR transactivation and ERK activation [20,24]. Recently, at least two different metalloproteinases, ADAM 10 or Kuzbanian [52] and ADAM 12 [53] have been implicated in mediating GPCR-induced EGFR transactivation in different model systems.

In the present study, we examined EGF receptor transactivation not only by assessing the overall tyrosine phosphorylation of this receptor in response to the GPCR agonists bombesin or LPA, but also by determining the phosphorylation of individual residues in the EGFR. A substantial literature has predominantly implicated either Src or MMPs in GPCR-induced EGFR transactivation [7,17–28]. Our approach allowed the examination of the interplay between these two mechanisms. Specifically, we report that addition of bombesin induced a marked increase in the phosphorylation of the EGFR at Tyr-845, a well established target of Src [29–33] and at Tyr-1068, a known autophosphorylation site [46,47]. An important feature of these results is that treatment with the broad-spectrum MMP inhibitor galardin prevented the phosphorylation of EGFR

Tyr-845 as effectively as it blocked that at Tyr-1068. This indicates that products of MMPs are necessary for Src-mediated phosphorylation of EGFR at Tyr-845 and thus, raise the attractive possibility that EGFR agonists generated by MMP-mediated cleavage of membrane precursors collaborate with Src kinases in promoting GPCR-induced EGFR transactivation. Since a recent report suggests that Src mediates MMP-dependent release of EGFR ligands [22], it is plausible that Src contributes to GPCR-induced EGFR transactivation at various steps of this mechanism of receptor cross-talk.

GPCR-induced EGFR transactivation leads to the assembly of signaling complexes that have been proposed to mediate Ras/ERK activation in response to GPCR activation [28]. However, the contribution of EGFR transactivation to ERK activation appears to depend on cellular context [17,48,54,55]. Here, we show that treatment with galardin prevented ERK activation induced by bombesin acting through ectopically expressed receptors in Rat-1 cells or by LPA acting via its endogenously expressed GPCRs in these cells. Thus, this provides an excellent cellular model system to examine the role of galardin-sensitive MMPs in late events, including GPCR-induced re-initiation of DNA synthesis, which has not been explored in any detail.

In this study, we demonstrate that galardin markedly attenuates (but does not eliminate) the stimulation of DNA synthesis in Rat-1 cells treated with either bombesin or LPA, indicating that MMP activity is necessary for maximal GPCR-induced DNA synthesis. Furthermore, addition of galardin up to 3 h after bombesin stimulation continued to inhibit DNA synthesis, suggesting that sustained MMP activity is required for this process. Our finding that MMPs play a role in GPCR-induced DNA synthesis is consistent with recent results demonstrating that the MMP inhibitors BB 2116 and batimastat reduced DNA synthesis induced by isoproterenol in adult rat cardiac fibroblasts [19] and LPA in squamous cell carcinoma cell lines of the head and neck [56], respectively.

It is noteworthy that galardin, at concentrations that completely abrogated bombesin-induced EGFR transactivation and ERK activation, produced only partial inhibition of DNA synthesis induced by either bombesin or LPA. These results imply that these agonists, which are known to activate multiple heterotrimeric G proteins, also stimulate mitogenic signaling through pathways independent of the EGFR. In line with this interpretation, recent work from our laboratory demonstrated that bombesin or LPA induce tyrosine phosphorylation of focal adhesion proteins through an EGFR-independent pathway [57].

In conclusion, the results presented here have important implications for understanding the role of the EGFR pathway in GPCR-induced mitogenic signaling. We demonstrate that (1) MMP products are necessary for Src-mediated phosphorylation of EGFR at Tyr-845, (2) sustained MMP activity is required for GPCR-induced DNA synthesis, and (3) MMPs generate the sustained release and buildup of EGFR ligands. Specifically, we propose that in Rat-1 cells the EGFR contributes to GPCR-induced cell cycle progression via sustained MMP-mediated release and accumulation of EGFR ligands, resulting in both ERK pathway activation and late response mitogenic signaling.

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