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GABA_BR-Induced EGFR Transactivation Promotes Migration of Human Prostate Cancer Cells[§]

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ABSTRACT

G protein-coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs) act in concert to regulate cell growth, proliferation, survival, and migration. Metabotropic GABA_B receptor (GABA_BR) is the GPCR for the main inhibitory neurotransmitter GABA in the central nervous system. Increased expression of GABA_BR has been detected in human cancer tissues and cancer cell lines, but the role of GABA_BR in these cells is controversial and the underlying mechanism remains poorly understood. Here, we investigated whether GABA_BR hijacks RTK signaling to modulate the fates of human prostate cancer cells. RTK array analysis revealed that the GABA_BR-specific agonist baclofen selectively induced the transactivation of EGFR in PC-3 cells. EGFR transactivation resulted in the activation of

ERK1/2 by a mechanism that is dependent on G_{i/o} protein and that requires matrix metalloproteinase-mediated proligand shedding. Positive allosteric modulators (PAMs) of GABA_BR, such as CGP7930, rac-BHFF, and GS39783, can function as PAM agonists to induce EGFR transactivation and subsequent ERK1/2 activation. Moreover, both baclofen and CGP7930 promoted cell migration and invasion through EGFR signaling. In summary, our observations demonstrated that GABA_BR transactivated EGFR in a ligand-dependent mechanism to promote prostate cancer cell migration and invasion, thus providing new insights into developing a novel strategy for prostate cancer treatment by targeting neurotransmitter signaling.

Introduction

Prostate cancer (PCa) is the second most frequently diagnosed cancer in men worldwide (Torre et al., 2015). Currently, few therapeutic options are available for patients with advanced PCa. Tyrosine kinase inhibitors, which are

effective in some human malignancies (Rask-Andersen et al., 2014), have been used as single agents or in combination with chemotherapy in clinical trials in patients with castration-resistant PCa; however, the results were not promising (Jakobovits, 2008; Gallick et al., 2012; Molife et al., 2014; Ojemuyiwa et al., 2014; Modena et al., 2016). Therefore, new mechanism-based inhibitors need to be developed to treat PCa patients. Since neurotransmitters have modulatory effects on tumor cells, the potential roles of receptors for neurotransmitters in tumors have attracted more and more research interest (Schuller, 2008a).

GABA is a main inhibitory neurotransmitter in the vertebrate central nervous system. Metabotropic GABA_B receptor (GABA_BR), a G protein-coupled receptor (GPCR) family member, is an obligatory heterodimer composed of GABA_{B1} and GABA_{B2} subunits. The extracellular domain of the GABA_{B1} subunit has a ligand-binding site for GABA, agonists,

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ABBREVIATIONS: AR, amphiregulin; CM, conditioned medium; DMSO, dimethylsulfoxide; EGF, epithelial growth factor; EGFR, epithelial growth factor receptor; ELISA, enzyme-linked immunosorbent assay; ERK1/2, extracellular signal-regulated kinase 1/2; FBS, fetal bovine serum; HB-EGF, heparin-binding epithelial growth factor; IGF-1R, insulin-like growth factor receptor 1; GABA_BR, metabotropic GABA_B receptor; GPCR, G protein-coupled receptor; HRP, horseradish peroxidase; MMP, matrix metalloproteinase; p, phosphorylated; PAM, positive allosteric modulator; PBS, phosphate-buffered saline; PCa, prostate cancer; PTX, pertussis toxin; RIPA, radioimmunoprecipitation assay; RT, room temperature; RTK, receptor tyrosine kinase; SFM, serum-free medium; TCL, total cell lysate; TGF- α , transforming growth factor- α ; TMB, tetramethylbenzidine.

or antagonists. Whereas the extracellular domain of the GABA_{B2} subunit lacks the ligand-binding capacities, the GABA_{B2} subunit is responsible for G_{i/o} protein coupling. It has been shown that specific allosteric modulators bind to GABA_{B2} subunit transmembrane domain (Bettler et al., 2004; Pinard et al., 2010).

Accumulated evidence suggests that GABA_BR is implicated in human cancers. The level of GABA_BR in thyroid and breast cancer specimens is positively correlated with tumor malignancy (Roberts et al., 2009; Jiang et al., 2012); moreover, GABA_BR increases the metastasis of mouse breast 4T1 cancer cells in vivo (Zhang et al., 2014). In contrast, the level of GABA_{B1} subunit in cholangiocarcinoma tissues is negatively correlated with the degree of cell differentiation, local invasion, and lymph node metastasis (Huang et al., 2013). Upregulated expression of the GABA_{B2} subunit is detected in female lung cancer patients with better outcome (Zhang et al., 2013), whereas the level of GABA_BR has no obvious correlation with the pathologic features of human gastric cancer (Zhu et al., 2004). In vitro studies reveal that GABA_BR agonists may inhibit (Fava et al., 2005; Wang et al., 2008; Huang et al., 2013; Zhang et al., 2013; Shu et al., 2016) or display no effect (Abdul et al., 2008; Lodewyckx et al., 2011; Zhang et al., 2014) on the proliferation of cancer cells. Activation of GABA_BR may promote (Azuma et al., 2003; Inamoto et al., 2007; Zhang et al., 2014), suppress (Fava et al., 2005; Schuller et al., 2008b,c; Lodewyckx et al., 2011), or have no influences (Joseph et al., 2002; Drell et al., 2003; Chen et al., 2012) on the migration and/or invasion of cancer cells. These observations suggest that the roles of GABA_BR in cancer cell proliferation, migration, and tumor malignancy are controversial.

It has been well documented that GPCRs induce the transactivation of various receptor tyrosine kinases (RTKs) including epithelial growth factor receptor (EGFR) family members, insulin-like growth factor receptor 1 (IGF-1R), fibroblast growth factor receptor 1, platelet-derived growth factor receptor, vascular endothelial growth factor receptor 2, TrkA/B, and c-Met (Wetzker and Böhmer, 2003; Tu et al., 2010; Cattaneo et al., 2014; Ryu et al., 2014). We have reported previously that specific activation of GABA_BR induced transactivation of IGF-1R in neurons (Tu et al., 2010; Lin et al., 2012). To investigate whether GABA_BR hijacks RTK signaling to modulate the fates of PCa cells, RTK array analysis was performed. The results showed that a specific GABA_BR agonist, baclofen, selectively induced transactivation of EGFR at multiple sites (Y845, Y1045, Y1068, and Y1086) in PC-3 cells, which was dependent on G_{i/o} protein and proligand shedding mediated by matrix metalloproteases (MMPs). Interestingly, positive allosteric modulators (PAMs) of GABA_BR, including CGP7930, rac-BHFF, and GS39783, can function as PAM agonists to induce EGFR transactivation and ERK1/2 activation. Moreover, both baclofen and CGP7930 promoted cell migration and invasion, which were dependent on EGFR transactivation. These observations suggest that GABA_BR-mediated EGFR signaling might be the potential targets in the treatment of advanced PCa.

Materials and Methods

Antibodies and Reagents

Primary antibodies including phosphorylated (p) ERK1/2 (T202/Y204), ERK1/2, pEGFR (Y845), pEGFR (Y992), pEGFR (Y998),

pEGFR (Y1045), pEGFR (Y1068), pEGFR (Y1086), pEGFR (Y1148), pEGFR (Y1173), EGFR, β -actin, EGFR antibody-conjugated sepharose beads, and horseradish peroxidase (HRP)-conjugated secondary antibodies against mouse and rabbit IgG were purchased from Cell Signaling Technology (Danvers, MA). Anti-phosphotyrosine antibody 4G10, 4G10-conjugated agarose beads, and EGFR-neutralizing antibody (LA1) were obtained from Millipore (Billerica, MA).

Pertussis toxin (PTX) and MMP inhibitor GM6001 [ilomastat (Galardin)] were purchased from Millipore. GABA_BR agonist baclofen, antagonist CGP54626, PAMs CGP7930, rac-BHFF, and GS39783 were obtained from Tocris Bioscience (Bristol, UK). EGFR inhibitors Tarceva (erlotinib) and Iressa (gefitinib) were from Active Biochem (Hong Kong, People's Republic of China).

Cell Culture

Human PCa cell line PC-3 was purchased from American Type Culture Collection (Manassas, VA). Cells were maintained in F-12 medium (ThermoFisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS; ThermoFisher Scientific), 100 units/ml penicillin, and 100 μ g/ml streptomycin (ThermoFisher Scientific) at 37°C with 5% CO₂. Cells were split every 3–4 days and medium was replaced every 2 days.

Treatment of Cells with Various Compounds

A total of 1.5×10^5 cells were seeded into 3.5-cm plates and grown in complete medium for 2 days at 37°C with 5% CO₂. Cells were then serum starved for 24 hours followed by incubation with u7 serum-free medium (SFM) for 30 minutes before being treated with various compounds, as described below.

GABA_BR Agonist. Cells were incubated with 200 μ M baclofen at 37°C for 0, 1, 5, 10, 20, 30, 60, and 120 minutes or with 0, 50, 100, 150, 200, 250, and 300 μ M baclofen at 37°C for 5 minutes.

PAMs of GABA_BR. CGP7930, rac-BHFF, and GS39783 were solubilized in dimethylsulfoxide (DMSO) with stock concentrations of 100, 100, and 10 mM, respectively. Cells were treated with 50 μ M CGP7930, 100 μ M rac-BHFF, or 10 μ M GS39783 at 37°C for 0, 1, 5, 10, 20, 30, 60, and 120 minutes or with various concentrations of CGP7930 (0, 3, 6, 12, 25, 50, and 100 μ M), rac-BHFF (0, 25, 50, 75, 100, 125, and 150 μ M), or GS39783 (0, 0.6, 1.2, 2.5, 5, 10, and 20 μ M) at 37°C for 10, 20, or 5 minutes, respectively. DMSO-treated cells were used as the control.

GABA_BR Antagonist. Cells were incubated with 20 μ M CGP54626 or 0.05% DMSO at 37°C for 30 minutes followed by incubation with 200 μ M baclofen, 50 μ M CGP7930, 100 μ M rac-BHFF, or 10 μ M GS39783 in the presence or absence of 20 μ M CGP54626 at 37°C for 5, 10, 20, or 5 minutes, respectively.

G_{i/o} Inhibitor. Cells were serum starved for 24 hours in the presence or absence of PTX (200 ng/ml) followed by treatment with 200 μ M baclofen in the presence or absence of PTX at 37°C for 5 minutes.

EGFR Inhibitors. Cells were incubated with 5 μ M Tarceva, 5 μ M Iressa, or 0.05% DMSO at 37°C for 8 hours. At the end of incubation, cells were treated with 200 μ M baclofen, 50 μ M CGP7930, 100 μ M rac-BHFF, or 10 μ M GS39783 in the presence or absence of Tarceva or Iressa at 37°C for 5, 10, 20, and 5 minutes, respectively.

MMP Inhibitor. Cells were pretreated with 10 μ M GM6001 or 0.05% DMSO at 37°C for 1 hour followed by incubation with 200 μ M baclofen in the presence or absence of GM6001 at 37°C for 5 minutes.

EGFR-Neutralizing Antibody. Cells were incubated with or without 2 μ g/ml EGFR-neutralizing antibody at 37°C for 1 hour followed by treatment with 200 μ M baclofen, 50 μ M CGP7930, 100 μ M rac-BHFF, or 10 μ M GS39783 in the presence or absence of neutralizing antibody at 37°C for 5, 10, 20, and 5 minutes, respectively.

At the end of each treatment, cells were lysed with radioimmuno-precipitation assay (RIPA) buffer containing proteinase inhibitors cocktail on ice for 30 minutes, sonicated for 30 seconds, and centrifuged at 4°C and 13,000 rpm for 10 minutes to remove cell debris. The

supernatants were transferred to new microtubes and stored at -20°C until use or subjected to SDS-PAGE immediately.

RTK Array Assay

Human RTK array was purchased from RayBiotech (Norcross, GA). The experiment was performed according to manufacturer instructions. Briefly, PC-3 cells grown in 10-cm plates were serum starved for 24 hours followed by treatment with or without 200 μM baclofen at 37°C for 5 minutes. Cells were then washed quickly with cold phosphate-buffered saline (PBS) and lysed immediately with cold RIPA buffer on ice for 30 minutes. Total cell lysates (TCLs) were sonicated for 30 seconds and centrifuged at 4°C and 13,000 rpm for 10 minutes to remove cell debris. Supernatants were diluted by blocking buffer in a final volume of 1.2 ml (300 $\mu\text{g}/\text{ml}$) and incubated with RTK array membranes, which were preincubated with blocking buffer overnight at 4°C . The membranes were washed three times (for 3 minutes each) with 2 ml of $1\times$ Wash Buffer I at room temperature (RT) with shaking, carefully transferred to a plastic container, and washed with a minimum of 20 ml of $1\times$ Wash Buffer I three times (for 5 minutes each) followed by washing with a minimum of 20 ml of $1\times$ Wash Buffer II three times (for 5 minutes each). The membranes were transferred to an eight-well tray and incubated with 1.2 ml of diluted biotin-conjugated antibody at RT with gentle shaking for 2 hours followed by incubation with 1.5 ml of $1\times$ HRP-conjugated streptavidin for 2 hours at RT, and washed with $1\times$ Wash Buffer I and II as described previously. Membranes were developed by using enhanced chemiluminescence reagents (ThermoFisher Scientific).

Immunoprecipitation

PC-3 cells grown in 10-cm plates were serum starved for 24 hours in the presence or absence of PTX (200 ng/ml) followed by treatment with or without 200 μM baclofen at 37°C for 5 minutes. Serum-starved PC-3 cells were also treated with or without 50 μM CGP7930, 100 μM rac-BHFF, or 10 μM GS39783 at 37°C for 10, 20, or 5 minutes, respectively. At the end of the treatment, cells were washed quickly with cold PBS and lysed immediately with cold RIPA buffer on ice for 30 minutes. TCLs were sonicated for 30 seconds and centrifuged at 4°C and 13,000 rpm for 10 minutes to remove cell debris. The supernatants (500 μg) were mixed with 20 μl of 4G10-conjugated agarose beads or EGFR antibody-conjugated sepharose beads in a final volume of 500 μl and incubated at 4°C overnight with continuous agitation. The beads were washed with RIPA buffer five times (600 μl each) followed by PBS two times, resuspended in 20 μl of $2\times$ electrophoresis loading buffer, boiled for 5 minutes, and centrifuged at 12,000 rpm for 5 minutes. The supernatants were transferred to new microtubes and subjected to immunoblot analysis.

Immunoblot Analysis

Equal amounts of TCLs were resolved by 10% SDS-PAGE gels followed by membrane transfer. Membranes were blocked with Tris-buffered saline containing 5% nonfat dry milk and 0.1% Tween-20 for 2 hours at RT and incubated with primary antibodies against EGFR [pEGFR (Y845), pEGFR (Y992), pEGFR (Y998), pEGFR (Y1045), pEGFR (Y1068), pEGFR (Y1086), pEGFR (Y1148), pEGFR (Y1173), ERK1/2, pERK1/2, or β -actin (1:2000-1:3000)] overnight at 4°C with continuous agitation. The next day, the membranes were washed and probed with proper HRP-conjugated secondary antibodies (1:20,000 to 1:30,000) at RT for 2 hours. Membranes were then washed and developed by using enhanced chemiluminescence reagents.

Enzyme-Linked Immunosorbent Assay

Human EGF, heparin-binding EGF (HB-EGF), amphiregulin (AR), and transforming growth factor- α (TGF- α) enzyme-linked immunosorbent assay (ELISA) kits were purchased from BOSTER (Pleasanton, CA). Serum-starved PC-3 cells seeded on 3.5-cm plates were treated with

200 μM baclofen at 37°C for 0, 5, 10, or 15 minutes, and conditioned media (CMs) were transferred to 1.5-ml microtubes. The 100- μl CMs or standards were added into 96-well plates and incubated at 37°C for 90 minutes followed by extensive washing. One hundred microliters of biotinylated antibodies were then added to the plates and incubated at 37°C for 60 minutes followed by three washes (for 1 minute each). One hundred microliters of avidin-biotin-peroxidase complex working solution was added to the plates except for the tetramethylbenzidine (TMB) blank well and incubated at 37°C for 30 minutes followed by five washes (for 1–2 minutes each). Ninety microliters of TMB developing reagent was added to the plate and incubated at 37°C in dark for 15–20 minutes. One hundred microliters of TMB stop solution was added to the plates, and optical density at 450 nm was measured in a microplate reader within 30 minutes. The experiments were performed in triplicate and repeated three times.

Transwell Assay

A modified Boyden chamber (Co-star; Corning, Rochester, NY) containing a polycarbonate membrane filter (6.5 mm in diameter with a pore size of 8 μm) was used in this assay. The upper chamber was coated with 70 μl of Matrigel (1 mg/ml; Corning) for invasion assay. Seven hundred microliters of F-12 medium supplemented with 10% FBS was placed in the lower chamber. Two hundred microliters of cell

Fig. 1. GABA_B-induced transactivation of EGFR in PC-3 cells. PC-3 cells grown in 10-cm plates with 80% confluence were serum starved for 24 hours and incubated with or without 200 μM baclofen at 37°C for 5 minutes. (A) Three hundred micrograms of TCL were subjected to RTK array assay as described in *Materials and Methods*. (B) Five hundred micrograms of TCL were immunoprecipitated with 4G10-conjugated agarose beads and probed with EGFR antibody. The levels of total EGFR, pEGFR (Y845), pEGFR (Y992), pEGFR (Y998), pEGFR (Y1045), pEGFR (Y1068), pEGFR (Y1086), pEGFR (Y1148), pEGFR (Y1173), and β -actin were assessed by immunoblot analysis (B) and quantitated by ImageJ (C). Data were normalized to total EGFR, expressed as the fold change (mean $\pm$ SEM; $n = 3$) and analyzed by paired t test. * $P < 0.05$; ** $P < 0.01$ (versus control). Bac, baclofen; Ctrl, control; IP, immunoprecipitation.

suspension (5×10^4 cells for migration, 1×10^5 cells for invasion) in the presence or absence of baclofen, CGP54626, CGP7930, or EGFR-neutralizing antibody was seeded into the upper chamber and incubated for 36 hours at 37°C in 5% CO_2 . Cells that remained on the top side of the filter membrane were gently removed with a cotton swab. Cells that migrated to or invaded the bottom surface of the filter were quickly fixed with methanol for 10 minutes and stained with 1% Crystal Violet/20% methanol for 20 minutes. The stained cells from at least eight random fields were examined under a microscope at a magnification of $200\times$. Images were captured by CellSens software and processed using Adobe Photoshop without changing the original appearance.

Statistical Analysis

Immunoblot results were quantitated by using ImageJ software, and the optical density of each band was normalized to EGFR, ERK1/2, or β -actin. Statistical analyses were performed by using GraphPad Prism 5 software. Data from three independent experiments were presented as the mean $\pm$ SEM. Statistical comparisons between groups were performed with paired *t* test or one-way ANOVA with post hoc test (Tukey). Data obtained from dose-response experiments were analyzed by using nonlinear curve fitting. Significance levels are indicated as follows: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Results

GABA_BR Agonist-Induced EGFR Transactivation in Human PCa Cells

The expression of GABA_BR was detected in human PCa cell lines PC-3 and LNCaP (Supplemental Fig. 1). To identify whether the activation of GABA_BR hijacks RTK signaling to modulate the fates of PCa cells, RTK array analysis was performed in PC-3 and LNCaP cells, which were stimulated with a specific GABA_BR agonist, baclofen (Supplemental Fig. 2A), for

5 minutes. The results showed that baclofen selectively upregulated the phosphorylation level of EGFR but not other RTKs in PC-3 cells (Fig. 1A).

To confirm the results of RTK array assay, PC-3 cell lysates prepared from untreated or baclofen-treated cells were immunoprecipitated with pTyr antibody 4G10 and immunoblotted with EGFR antibody. As shown in Fig. 1, B and C, baclofen increased the total phosphorylation level of EGFR for 2.063 ± 0.627 -fold ($P < 0.05$, $n = 3$). Upon agonist stimulation, Y845 in the kinase domain and eight tyrosine residues in the C-tail of EGFR (Y974, Y992, Y1045, Y1068, Y1086, Y1101, Y1148, and Y1173) could be phosphorylated (Conte and Sigismund, 2016). Analysis of single-site phosphorylation of EGFR revealed that baclofen significantly upregulated the levels of pY845, pY1045, pY1068, and pY1086 for 0.490 ± 0.101 -fold ($P < 0.05$, $n = 3$), 0.481 ± 0.052 -fold ($P < 0.01$, $n = 3$), 0.883 ± 0.221 -fold ($P < 0.05$, $n = 3$), and 1.070 ± 0.356 -fold ($P < 0.05$, $n = 3$), respectively, but showed no obvious influence on the phosphorylation of Y1148 and Y1173 (Fig. 1, B and C). Phosphorylation of Y992 and Y998 was not detected in the presence of baclofen (data not shown), and phosphorylation of Y974 and Y1101 was not analyzed due to a lack of antibodies.

GABA_BR Agonist-Induced Activation of ERK1/2 in Human PCa Cells

GPCR-induced EGFR transactivation can activate ERK1/2 signaling cascades (Xiao et al., 2003; Zhou et al., 2012; George et al., 2013; Lai et al., 2016). To examine whether EGFR transactivation by GABA_BR induced ERK1/2 activation, PC-3 cells were treated with baclofen or GABA for various period of

Fig. 2. Baclofen induced ERK1/2 activation in a time- and dose-dependent manner. PC-3 cells seeded in 3.5-cm plates with 80% confluence were serum starved for 24 hours, replaced with SFM, and incubated at 37°C for 30 minutes. (A) Cells were treated with 200 μM baclofen at 37°C for 0, 1, 5, 10, 20, 30, 60, or 120 minutes. (B) Cells were incubated with 0, 50, 100, 150, 200, 250, or 300 μM baclofen at 37°C for 5 minutes. (C) Cells were pretreated with 20 μM CGP54626 or 0.05% DMSO at 37°C for 30 minutes followed by incubation with or without 200 μM baclofen at 37°C for 5 minutes. Cells were incubated with 5 μM Tarceva (D) or 5 μM Iressa (E) at 37°C for 8 hours and then treated with 200 μM baclofen at 37°C for 5 minutes. Cells treated with 0.05% DMSO were used as controls. At the end of incubation, equal amounts of TCLs were subjected to immunoblot analysis. The levels of pERK1/2 were normalized to total ERK1/2 and expressed as the fold change (mean $\pm$ SEM; $n = 3$). Data were analyzed using nonlinear curve fitting (B) or one-way ANOVA with post hoc test (Tukey) (C–E). *** $P < 0.001$. Conc, concentration; ctrl, control; ns, not significant.

time. The results showed that both baclofen and GABA induced a fast and transient phosphorylation of ERK1/2 in a time-dependent manner, which peaked at 5 minutes and returned to basal level after 60 minutes (Fig. 2A; Supplemental Fig. 3). Baclofen also displayed a dose-dependent effect on ERK1/2 activation, with a minimum concentration of 200 μ M (Fig. 2B).

Pretreatment of PC-3 cells with CGP54626 (Supplemental Fig. 2B), a high-affinity and highly selective GABA_BR antagonist (Filip and Frankowska, 2008), abrogated baclofen-

induced ERK1/2 phosphorylation (Fig. 2C), indicating that ERK1/2 activation was specifically mediated by GABA_BR.

Tarceva (also named erlotinib) and Iressa (also named gefitinib) are small-molecule inhibitors of EGFR (Sharma et al., 2007). Pretreatment of PC-3 cells with Tarceva or Iressa completely blocked baclofen-induced ERK1/2 phosphorylation (Fig. 2, E and F), suggesting that baclofen-induced ERK1/2 activation was dependent on EGFR transactivation by GABA_BR.

Fig. 3. EGFR transactivation induced by baclofen was dependent on $G_{i/o}$ protein and MMPs. (A) PC-3 cells grown in 10-cm plates with 80% confluence were serum starved for 24 hours in the presence or absence of 200 ng/ml PTX. Cells were then incubated with or without 200 μ M baclofen at 37°C for 5 minutes. Five hundred micrograms of TCL were immunoprecipitated with EGFR-conjugated agarose beads and probed with EGFR antibody. The levels of total EGFR, pEGFR (Y845), pEGFR (Y1045), pEGFR (Y1068), pEGFR (Y1086), and β -actin were assessed by immunoblot analysis (A) and quantitated (B). (C) The levels of pERK1/2, ERK1/2, and β -actin in samples prepared in (A) were analyzed by immunoblot assay. (D) Serum-starved cells grown in 3.5-cm plates were preincubated with 20 μ M GM6001 (GM) or 0.05% DMSO at 37°C for 1 hour and then treated with 200 μ M baclofen at 37°C for 5 minutes. The levels of total EGFR, pEGFR (Y845), pEGFR (Y1045), pEGFR (Y1068), pEGFR (Y1086), and β -actin were assessed by immunoblot analysis (D) and quantitated (E). (F) The levels of pERK1/2, ERK1/2, and β -actin in samples prepared in (D) were analyzed by immunoblot assay. (G) Serum-starved cells grown in 3.5-cm plates were preincubated with or without EGFR-neutralizing antibody (2 μ g/ml) at 37°C for 1 hour and then were treated with 200 μ M baclofen at 37°C for 5 minutes. Equal amounts of TCLs were subjected to immunoblot analysis to assess the levels of pERK1/2, ERK1/2, and β -actin. (H) Serum-starved cells grown in 3.5-cm plates were treated with 200 μ M baclofen at 37°C for 0, 5, 10, or 15 minutes. CMs were then collected and the levels of EGF and HB-EGF were analyzed by ELISA, which were performed in triplicate and repeated three times. Data were expressed as the fold change (mean $\pm$ SEM; $n = 3$) and analyzed by one-way ANOVA with post hoc test (Tukey) (B, C, E–G) or paired t test (H). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Bac, baclofen; Ctrl, control; ns, not significant.

EGFR Transactivation by GABA_BR Agonist Was Mediated by G_{i/o} Protein and Ligand-Dependent Pathway

GABA_BR is coupled to G_{i/o} protein (Filip and Frankowska, 2008). Pretreatment of cells with PTX, an inhibitor of G_{i/o} protein, suppressed baclofen-induced total and site-specific phosphorylation of EGFR and ERK1/2 phosphorylation (Fig. 3, A–C), implicating that baclofen-induced EGFR transactivation and subsequent ERK1/2 activation were dependent on G_{i/o} protein.

EGFR transactivation by GPCRs is mediated by either a ligand-dependent or a ligand-independent pathway. Ligand-dependent RTK transactivation by GPCRs is dependent on the activation of MMPs (Cattaneo et al., 2014). EGFR ligands,

including EGF, HB-EGF, AR, TGF- α , epiregulin, betacellulin, and epigen, are synthesized as transmembrane proligands that are later processed by MMPs (Harris et al., 2003; Cattaneo et al., 2014). GM6001, a pan-MMP inhibitor, abolished baclofen-induced multisite phosphorylation of EGFR and ERK1/2 activation (Fig. 3, D–F), suggesting that baclofen-induced EGFR transactivation was dependent on MMPs.

MMPs have been reported to mediate the shedding of pro-HB-EGF, pro-AR, or pro-TGF- α from the cell surface and subsequent EGFR transactivation (Zhao et al., 2004; Kamanga-Sollo et al., 2014; Moody et al., 2014; Light and Hammes, 2015; Overland and Insel, 2015). EGFR-neutralizing antibody, which blocked the ligand-binding site of EGFR, abrogated

Fig. 4. Baclofen (Bac) promoted the migration of PC-3 cells. Seven hundred microliters of medium containing 10% FBS was added in the lower chamber of the transwell. PC-3 cells grown in 10-cm plates were serum starved for 24 hours and resuspended in SFM. (A) A total of 5×10^4 cells/200 μ l in the presence of 0, 50, 100, or 200 μ M baclofen were added into the upper chamber and incubated for 36 hours at 37°C and 5% CO₂. (C) A total of 5×10^4 cells/200 μ l in the presence or absence of 100 μ M baclofen or 5 μ M CGP54626 (CGP) were transferred to the upper chamber and incubated for 36 hours at 37°C and 5% CO₂. (E) A total of 5×10^4 cells/200 μ l in the presence or absence of 100 μ M baclofen or EGFR-neutralizing antibody (Ab) (0.5 μ g/ml) were added to the upper chamber and incubated for 36 hours at 37°C and 5% CO₂. At the end of incubation, cells remained on the upper side were fixed, stained, and observed under a microscope. Images from at least eight random fields were captured by CellSens software. The numbers of migrated cells in (A), (C), and (E) were counted and expressed as the mean $\pm$ SEM ($n = 8$) in (B), (D), and (F), respectively. The experiments were performed three times, and the results were reproducible. One representative experiment was shown in each panel. Data were analyzed by paired t test (B) or one-way ANOVA with post hoc test (Tukey) (D) and (F). *** $P < 0.001$. Scale bar, 200 μ m. Ctrl, control; FV, field of view; ns, not significant.

baclofen-induced ERK1/2 phosphorylation in PC-3 cells (Fig. 3G). To identify which EGFR proligands were released by MMPs from the cell surface, ELISA experiments were performed. As shown in Fig. 3H, the mean basal EGF level in the control group was 3.177 ± 0.292 pg/ml ($n = 3$), and baclofen treatment of 5, 10, or 15 minutes increased the EGF level to 4.008 ± 0.214 pg/ml ($P < 0.05$, $n = 3$), 4.888 ± 0.361 pg/ml ($P < 0.05$, $n = 3$) and 6.056 ± 0.235 pg/ml ($P < 0.05$, $n = 3$), respectively. Baclofen had no significant influence on HB-EGF level until the 15-minute treatment, whereas no upregulation of TGF- α and AR levels was detected in the presence of baclofen (data not shown). These data indicated that the production of EGF but not of HB-EGF was upstream of ERK1/2 phosphorylation upon GABA_BR activation; therefore, MMP-mediated shedding of pro-EGF might be involved in baclofen-induced EGFR signaling in PC-3 cells.

GABA_BR Agonist Promoted Migration and Invasion of PC-3 Cells

Baclofen has been shown to enhance the invasive ability of human prostate C4-2 cancer cells (Azuma et al., 2003). To analyze the effects of baclofen on the migration and invasion of PC-3 cells, transwell experiments were performed. In the control group, the number of migrated cells was 105.8 ± 9.176 per field

of view ($n = 8$), which increased to 167.6 ± 7.573 ($P < 0.001$, $n = 8$), 204.5 ± 5.292 ($P < 0.001$, $n = 8$), and 220.3 ± 9.080 ($P < 0.001$, $n = 8$) in the presence of 50, 100, and 200 μ M baclofen, respectively (Fig. 4, A and B). GABA_BR antagonist CGP54626 (5 μ M) suppressed baclofen (100 μ M)-induced migration and invasion (Fig. 4, C and D; Fig. 5, A and B). Transwell experiments were then performed in the presence or absence of EGFR neutralizing antibody to explore the role of GABA_BR-induced ligand-dependent EGFR transactivation in migration and invasion. The results showed that EGFR-neutralizing antibody remarkably suppressed baclofen-induced migration and invasion (Fig. 4, E and F; Fig. 5, C and D), suggesting that ligand-dependent transactivation of EGFR by baclofen was essential for the migration and invasion of PC-3 cells.

As a control, Baclofen displayed no effect on the survival of PC-3 cells after 96 hours of treatment. However, CGP54626 inhibited cell survival in a time- and dose-dependent manner, and 40 μ M CGP54626 significantly inhibited cell survival after 48 hours of treatment (Supplemental Fig. 4).

PAMs of GABA_BR Directly Induced ERK1/2 Activation as PAM Agonists

CGP7930, rac-BHFF, and GS39783, the PAMs of GABA_BR (Supplemental Fig. 2C), are capable of modulating the efficacy

Fig. 5. Baclofen (Bac) promoted the invasion of PC-3 cells. Seven hundred microliters of medium-supplemented 10% FBS was added in the lower chamber, and the filter of the transwell was coated with 70 μ l of Matrigel (1 mg/ml). PC-3 cells grown in 10-cm plates were serum starved for 24 hours and resuspended in SFM. (A) A total of 1×10^5 cells/200 μ l in the presence or absence of 100 μ M baclofen or 5 μ M CGP54626 (CGP) were transferred to the upper chamber and incubated for 36 hours at 37°C in 5% CO₂. (C) A total of 1×10^5 cells/200 μ l in the presence or absence of 100 μ M baclofen or EGFR-neutralizing antibody (Ab) (0.5 μ g/ml) were added to the upper chamber and incubated for 36 hours at 37°C in 5% CO₂. The cells that remained on the upper side of the filters were fixed, stained, and observed under a microscope. Images from at least eight random fields were captured by CellSens software. The numbers of invasive cells in (A) and (C) were counted and expressed as the mean $\pm$ SEM ($n = 8$) in (B) and (D), respectively. The experiments were performed three times, and the results were reproducible. One representative experiment was shown in each panel, and statistical analysis was performed using one-way ANOVA with post hoc test (Tukey). *** $P < 0.001$. Scale bar, 200 μ m. Ctrl, control; FV, field of view; ns, not significant.

of GABA_BR-induced signal transduction (Urwyler et al., 2001, 2003, 2005; Malherbe et al., 2008). PAM agonist activities of these three compounds have been reported previously (Binet et al., 2004; Tu et al., 2007, 2010; Malherbe et al., 2008; Gjoni

and Urwyler, 2009; Koek et al., 2010; Gannon and Millan, 2011; Sun et al., 2016). To analyze whether these PAMs have intrinsic activity in cancer cells, PC-3 cells were incubated with PAMs without the addition of an agonist. As shown in

Fig. 6. GABA_B PAMs induced ERK1/2 activation in PC-3 cells. Cells seeded in 3.5-cm plates with 80% confluence were serum starved for 24 hours, replaced with SFM, and incubated at 37°C for 30 minutes. (A) Cells were treated with 50 μ M CGP7930 at 37°C for 0, 1, 5, 10, 20, 30, 60, or 120 minutes. (B) Cells were incubated with 0, 3, 6, 12, 25, 50, or 100 μ M CGP7930 at 37°C for 10 minutes. (C) Cells were pretreated with 20 μ M CGP54626 or 0.05% DMSO at 37°C for 30 minutes followed by incubation with or without 50 μ M CGP7930 at 37°C for 10 minutes. (D) Cells were treated with 100 μ M rac-BHFF at 37°C for 0, 1, 5, 10, 20, 30, 60, or 120 minutes. (E) Cells were incubated with 0, 25, 50, 75, 100, 125, or 150 μ M rac-BHFF at 37°C for 20 minutes. (F) Cells were pretreated with 20 μ M CGP54626 or 0.05% DMSO at 37°C for 30 minutes followed by incubation with or without 100 μ M rac-BHFF at 37°C for 20 minutes. (G) Cells were incubated with 10 μ M GS39783 at 37°C for 0, 1, 5, 10, 20, 30, 60, or 120 minutes. (H) Cells were treated with 0, 0.6, 1.2, 2.4, 5, 10, or 20 μ M GS39783 at 37°C for 5 minutes. (I) Cells were pretreated with 20 μ M CGP54626 or 0.05% DMSO at 37°C for 30 minutes followed by incubation with or without 10 μ M GS39783 at 37°C for 5 minutes. At the end of incubation, equal amounts of TCLs were resolved by SDS-PAGE followed by immunoblot analysis; the levels of pERK1/2 were normalized to total ERK1/2 and expressed as the fold change (mean $\pm$ SEM; $n = 3$). Statistical analysis was performed using nonlinear curve fitting (B, E, and H) or one-way ANOVA with post hoc test (Tukey) (C, F, and I). $^{**}P < 0.01$; $^{***}P < 0.001$. Conc, concentration; ns, not significant.

Fig. 6, A and B, CGP7930 can act as a PAM agonist to induce ERK1/2 phosphorylation in a time- and dose-dependent manner. Compared with baclofen-induced transient activation of ERK1/2, CGP7930 induced a sustained phosphorylation of ERK1/2, which peaked at 10 minutes, and lasted for more than 2 hours. Twelve micromolar CGP7930 was sufficient to activate ERK1/2, and 50 μ M CGP7930 resulted in a maximum activation of ERK1/2 with an EC₅₀ value of 20.56 μ M. The profile of ERK1/2 phosphorylation induced by rac-BHFF was similar to that induced by CGP7930, which was sustained, peaking at 20 minutes and lasting for more than 2 hours. The minimum concentration of rac-BHFF to activate ERK1/2 was 75 μ M (Fig. 6, D and E). ERK1/2 activation-induced

GS39783 was similar to that induced by baclofen, which was transient, peaked at 5 minutes, and returned to basal level after 30 minutes. A GS39783 concentration of 0.6 μ M was sufficient to induce ERK1/2 phosphorylation, and a GS39783 concentration of 5 μ M resulted in maximum phosphorylation of ERK1/2 with an EC₅₀ value of 4.85 μ M (Fig. 6, G and H).

It has been documented that PC-3 cells may synthesize endogenous GABA (Ippolito and Piwnicka-Worms, 2014). To clarify whether ERK1/2 activation induced by PAMs was due to a direct agonistic effect or the potentiation of endogenously produced GABA, PC-3 cells were pretreated with GABA_BR antagonist CGP54626 followed by PAMs. The results showed

Fig. 7. GABA_BR PAMs induced EGFR signaling in PC-3 cells. PC-3 cells grown in 10-cm plates with 80% confluence were serum starved for 24 hours and incubated with or without 50 μ M CGP7930 (A), 100 μ M rac-BHFF (B), or 10 μ M GS39783 (GS) (C) at 37°C for 10, 20, or 5 minutes, respectively. A total of 500 μ g of TCL was immunoprecipitated with 4G10-conjugated agarose beads and probed with EGFR antibody. The levels of total EGFR, pERK1/2, ERK1/2, and β -actin were analyzed by immunoblot assay. Cells seeded in 3.5-cm plates with 80% confluence were serum starved for 24 hours and pretreated with 5 μ M Tarceva (D–F), 5 μ M Iressa (G–I), or 2 μ g/ml EGFR-neutralizing antibody (J–L) at 37°C for 8, 8, or 1 hour, respectively. Cells were then incubated with 50 μ M CGP7930 (D, G, and J), 100 μ M rac-BHFF (E, H, and K), or 10 μ M GS39783 (F, I, and L) at 37°C for 10, 20, or 5 minutes, respectively. The levels of pERK1/2, ERK1/2, and β -actin were analyzed by immunoblot assay and quantitated. Data were normalized to total EGFR or ERK1/2, expressed as the fold change (mean $\pm$ SEM; $n = 3$) and analyzed by paired t test (A–C) or one-way ANOVA with post hoc test (Tukey) (D–L). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Ctrl, control; IP, immunoprecipitation; ns, not significant.

that CGP54626 did not abrogate ERK1/2 activation induced by CGP7930 and rac-BHFF (Fig. 6, C and F), suggesting that CGP7930 and rac-BHFF had a direct agonistic effect on ERK1/2 activation. GS39783-induced activation of ERK1/2 was suppressed by CGP54626 (Fig. 6I), indicating that GS39783 may function as a pure PAM in PC-3 cells.

PAMs of GABA_BR-Stimulated EGFR Signaling in PC-3 Cells

To investigate whether CGP7930, rac-BHFF, and GS39783 can activate EGFR signaling in PC-3 cells, immunoprecipitation

experiments were performed. The results showed that these three PAMs significantly increased the total phosphorylation level of EGFR (Fig. 7, A–C). Further analysis showed that ERK1/2 phosphorylation induced by CGP7930, rac-BHFF, and GS39783 was suppressed by EGFR inhibitors Tarceva (Fig. 7, D–F), Iressa (Fig. 7, G and H), and EGFR-neutralizing antibody (Fig. 7, J and L). These results suggested that PAMs of GABA_BR functioned in a manner similar to baclofen to induce EGFR transactivation and EGFR ligand-dependent ERK1/2 activation.

Fig. 8. CGP7930 promoted the migration and invasion of PC-3 cells. Seven hundred microliters of medium containing 10% FBS was added to the lower chamber of the transwell. PC-3 cells grown in 10-cm plates were serum starved for 24 hours and resuspended in SFM. (A) A total of 5×10^4 cells/200 μ l in the presence of 0, 1, 2, or 4 μ M CGP7930 was transferred to the upper chamber and incubated for 36 hours at 37°C in 5% CO₂. (C) A total of 5×10^4 cells/200 μ l in the presence or absence of 4 μ M CGP7930 or 0.5 μ g/ml EGFR-neutralizing antibody were added to the upper chamber and incubated for 36 hours at 37°C in 5% CO₂. (E) The filter of the transwell was coated with 70 μ l of Matrigel (1 mg/ml). A total of 1×10^5 cells/200 μ l in the presence or absence of 4 μ M CGP7930 or 0.5 μ g/ml EGFR-neutralizing antibody were added to the upper chamber and incubated for 36 hours at 37°C in 5% CO₂. Images of cell migration from at least eight random fields were captured by CellSens software. The numbers of migrated or invaded cells in (A), (C), and (E) were counted and expressed as the mean $\pm$ SEM ($n = 8$) in (B), (D), and (F), respectively. The experiments were performed three times, and the results were reproducible. One representative experiment was shown in each panel. Statistical analysis was performed using paired t test (B) or one-way ANOVA with post hoc test (Tukey) (D–F). ** $P < 0.01$; *** $P < 0.001$. Ab, antibody; Ctrl, control; FV, field of view; ns, not significant. Scale bar, 200 μ m.

CGP7930 Promoted Migration and Invasion of PC-3 Cells

Based on the time and dose effects of CGP7930 on cell survival (data not shown), transwell experiments were performed in the presence of maximal 4 μ M CGP7930. As shown in Fig. 8, A and B, CGP7930 promoted the migration of PC-3 cells in a dose-dependent manner, and 1 μ M CGP7930 was sufficient to enhance cell migration.

Transwell experiments were also performed in the presence or absence of EGFR-neutralizing antibody to explore the role of CGP7930-induced EGFR transactivation in migration and invasion. The results showed that EGFR-neutralizing antibody blocked CGP7930-induced migration (Fig. 8, C and D) and invasion (Fig. 8, E and F), suggesting that ligand-dependent transactivation of EGFR by CGP7930 was important for the migration and invasion of PC-3 cells.

Discussion

The EGFR family has received much attention in pharmacological research due to their strong association with malignant proliferation (Gallick et al., 2012). In addition to direct activation by specific ligands, EGFR family members could also be transactivated by a variety of GPCR agonists (Daub et al., 1996; Zhou et al., 2012; Cattaneo et al., 2014; Muñoz-Moreno et al., 2014; Lai et al., 2016; Wang, 2016). In this study, we demonstrated for the first time that specific activation of GABA_BR, a neurotransmitter receptor belonging to the GPCR family, selectively induced EGFR transactivation in PC-3 cells. Published data revealed that EGFR ligands can induce multisite phosphorylation of EGFR at Y845, Y974, Y992, Y1045, Y1068, Y1086, Y1101, Y1148, and Y1173 (Conte and Sigismund, 2016). Single-site phosphorylation of EGFR at Y845 or Y992 has been detected in PCa cells treated with neuropeptides (GPCR agonists) (Amorino et al., 2007; DaSilva et al., 2013). Dual-site phosphorylation of EGFR at Y845 and

Y1068 was observed in rat-1 cells stimulated with bombesin and lysophosphatidic acid (GPCR agonists) (Santiskulvong and Rozengurt, 2003). We identified that baclofen selectively induced the multisite phosphorylation of EGFR at Y845, Y1045, Y1068, and Y1086. p-Y1045, p-Y1068, and p-Y1086 in EGFR are involved directly or indirectly in the recruitment of Cbl to EGFR, leading to ubiquitination of EGFR. Extensively ubiquitinated EGFR might be internalized through non-clathrin endocytosis and degraded in lysosomes (Conte and Sigismund, 2016). It is of particular interest that baclofen selectively induced multisite phosphorylation of tyrosine residues that are involved in EGFR ubiquitination. It is worth knowing whether baclofen induces massive ubiquitination and non-clathrin endocytosis biased internalization of EGFR (compared with classic clathrin-mediated endocytosis) in PC-3 cells.

EGFR transactivation by GPCRs occurs mainly through ligand-dependent and ligand-independent mechanisms. In the ligand-dependent mechanism, MMPs mediate the shedding of proligands from cell surface and subsequent RTK transactivation by GPCRs (Cattaneo et al., 2014). It has been reported that MMPs mediated the shedding of pro-HB-EGF and subsequent EGFR transactivation in PC-3 cells treated with neurotensin, bombesin, tetradecanoyl-phorbol-13-acetate, or calcium-sensing receptor stimulus (increased level of extracellular Ca²⁺) (Prenzel et al., 1999; Madarame et al., 2003; Hassan et al., 2004; Yano et al., 2004). Our study suggested that MMPs may promote the cleavage of pro-EGF from cell surface and EGFR transactivation in PC-3 cells stimulated with baclofen. A significant increase of HB-EGF level was detected 10 minutes later than that of pro-EGF. Therefore, EGF might be the main ligand that induces the fast transactivation of EGFR. AR and TGF- α were not detected in this study, probably due to the lack of expression or shedding of these two ligands or to the fact that the concentrations of these two ligands were too low to be detected.

Fig. 9. Diagram of GABA_BR-induced EGFR transactivation in PC-3 cells. Upon agonist binding, GABA_BR activates G_{i/o} protein, and MMP-mediated cleavage of pro-EGF from cell surface leads to multisite phosphorylation of EGFR at Y845, Y1045, Y1068, Y1086, and subsequent ERK1/2 activation. In addition to baclofen, PAMs of GABA_BR (CGP7930, rac-BHFF, and GS39783) can function as PAM agonists to induce EGFR transactivation and increase ERK1/2 activity. Both baclofen and CGP7930 promote cell migration and invasion in an EGFR transactivation-dependent pathway. GB1, GABA_{B1} subunit; GB2, GABA_{B2} subunit.

We have reported previously that GABA_BR induced the transactivation of IGF-1R in neurons in a ligand-independent pathway, which was mediated by phospholipase C, cytosolic Ca²⁺, and focal adhesion kinase 1 (Tu et al., 2010); whereas, this study demonstrated that GABA_BR induced EGFR transactivation in a ligand-dependent pathway, indicating that a single GPCR may transactivate two RTKs by different mechanisms, which might be tissue specific. So far, most studies have suggested one GPCR to one RTK transactivation pathway (Cattaneo et al., 2014; Wang, 2016). However, similar to our results, several other studies suggested a single GPCR to two RTKs pathway. In PC-3 and LNCaP cells, neuropeptides induced Src- and MMP-dependent EGFR transactivation (pY92) and subsequent IGF-1R phosphorylation (DaSilva et al., 2013). In PC-3 cells, growth hormone-related hormone stimulated a rapid (30-second) ligand-independent activation of EGFR and HER2, and a slow (30-minute) ligand-dependent activation of EGFR and HER2 (Muñoz-Moreno et al., 2014). In primary cultures of Müller cells of the guinea pig, P2Y receptor agonist ATP induced transactivation of platelet-derived growth factor receptor and EGFR in a ligand-dependent pathway (Milenkovic et al., 2003). Therefore, GPCR agonists may induce a more global RTK response, depending on cellular context.

ERK1/2 signaling cascade, one of the overlapping signaling pathways downstream of GPCRs and RTKs, is involved in the development and progression of various human cancers (Osborne et al., 2012). This study revealed that both agonists and PAMs of GABA_BR can induce ERK1/2 activation in an EGFR ligand-dependent pathway. Interestingly, CGP7930- and rac-BHFF-induced ERK1/2 activation was not antagonized by GABA_BR antagonist CGP54626, suggesting a direct agonist effect of these two compounds, which is consistent with data published by us and other research groups (Binet et al., 2004; Tu et al., 2007, 2010; Malherbe et al., 2008; Koek et al., 2010; Sun et al., 2016). ERK1/2 activation induced by GS39783 was blocked by CGP54626, indicating that GS39783 may function as a pure PAM in PC-3 cells. But we did not exclude the possibility that GS39783 may have a direct agonist activity. As described in *Materials and Methods*, serum-starved PC-3 cells were washed with SFM before PAM treatment, so the endogenous level of GABA in PC-3 cells might be too low to be effective. The different effects of CGP54626 on ERK1/2 activation induced by CGP7930, rac-BHFF, and GS39783 suggested that GABA_BR PAMs may have distinct binding sites on the transmembrane domain of the GABA_{B2} subunit. Upon CGP54626 binding, both GABA_{B1} and GABA_{B2} subunits undergo a conformational change, which may distort the binding site for GS39783 but retain the binding capacity for CGP7930 and rac-BHFF.

It has been reported that GABA simulated proliferation of PC-3 cells (Wu et al., 2014). In this study, baclofen displayed no effect on the survival of PC-3 cells, but GABA_BR antagonist CGP54626 inhibited cell survival. Therefore, the effect of baclofen on the survival of PC-3 cells was probably masked by endogenous GABA: when the effect of GABA was blocked by GABA_BR antagonist, cell survival was suppressed.

This study showed that both baclofen and CGP7930 significantly promoted the migration and invasion of PC-3 cells, but EGFR-neutralizing antibody abolished such effects, indicating that GABA_BR-induced EGFR transactivation was essential for the enhanced migration and invasion of PC-3 cells. Similar results were obtained in PCa cells C4-2, renal cell carcinoma

cells, and mouse 4T1 cells treated with GABA or baclofen (Azuma et al., 2003; Inamoto et al., 2007; Zhang et al., 2014).

Taken together, our study revealed that GABA_BR agonists selectively induced multisite phosphorylation of EGFR at Y845, Y1045, Y1068, and Y1086, which was dependent on G_{i/o} protein—and MMP-mediated cleavage of pro-EGF, resulting in ERK1/2 activation. PAMs of GABA_BR (CGP7930, rac-BHFF, and GS39783) can function as PAM agonists to induce EGFR transactivation and subsequent ERK1/2 activation. Baclofen- and CGP7930-induced EGFR transactivation played an important role in the migration and invasion of PC-3 cells (Fig. 9). To our knowledge, this is the first time the mechanism of GABA_BR-induced EGFR signaling in cancer cells has been identified. Since clinical trials of EGFR inhibitors in patients with castration-resistant PCa produced disappointing results (Jakobovits, 2008; Gallick et al., 2012; Molife et al., 2014; Ojemuyiwa et al., 2014), neurotransmitter GABA and its receptor GABA_BR might be potential targets in the clinical treatment of PCa patients.

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Authorship Contributions

Participated in research design: Jiang, Liu.
Conducted experiments: Xia, He, Zhu, Wang, Li, Zhang, Jiang.
Performed data analysis: Xia, He, Zhu, Wang, Li, Jiang.
Wrote or contributed to the writing of the manuscript: Xia, He, Jiang, Liu.

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