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ORIGINAL ARTICLE

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Signal transduction through substance P receptor in human glioblastoma cells: roles for Src and PKCδ

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Abstract Substance P receptor (SPR), a G protein-coupled receptor (GPCR), is found in human glioblastomas, and has been implicated in their growth. Consistent with a role for SPR in cell growth, activation of SPR in U373 MG human glioblastoma cells leads to the phosphorylation of mitogen-activated protein kinases [extracellular signal-regulated kinase 1 and 2 (ERK1/2)] and stimulation of cell proliferation. The purpose of the present study was to elucidate the pathway through which these actions occur. Using either the epidermal growth factor receptor (EGFR) kinase inhibitor, AG 1478, or a small-interfering RNA (siRNA) directed against human EGFR, we found that transactivation of EGFR by SPR is only marginally involved in SP-dependent ERK1/2 phosphorylation. Src, however, is shown to be a major component of SPR signaling because the Src kinase inhibitor, PP2, and a kinase-dead Src mutant both inhibit SP-dependent ERK1/2 phosphorylation. We also report that SPR stimulates the phosphorylation of protein kinase Cδ(PKCδ), and that this stimulation is blocked by PP2. SP-dependent ERK1/2 phosphorylation is also blocked by rottlerin, a PKCδ inhibitor, and the calcium scavenger, BAPTA/AM. Finally, rottlerin and PP2 were both found to inhibit the growth of several glioblastoma cell lines, underscoring the potential of these agents to block glioblastoma growth.

Keywords Substance P receptor · Epidermal growth factor receptor (EGFR) · Src · ERK1/2 · Protein kinase C (PKC) · Glioblastoma

Abbreviations SPR: Substance P receptor · GPCR: G protein-coupled receptor · SP: Substance P · PKC: Protein kinase C · ERK1/2: Extracellular signal-regulated kinase 1 and 2 · EGFR: Epidermal growth factor receptor · BAPTA/AM: 1,2-*bis*-(*o*-Aminophenoxy)ethane-*N*, *N*, *N*′, *N*′-tetraacetic acid tetra-(acetoxymethyl) ester · PP2: 4-Amino-5-(4-chlorphenyl)-7-(*t*butyl) pyrazolo[3,4-*d*]pyrimidine · PP3: 4-Amino-7-phenylpyrazol[3,4-*d*]pyrimidine · EGF: Epidermal growth factor · PMA: Phorbol-12-myristate-13-acetate · GF109203X: Bisindoylmaleimide I

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Introduction

Substance P receptor (SPR; also known as NK1 receptor), a G protein-coupled receptor (GPCR), mediates the effects of substance P (SP). Although a role for SPR in pain and inflammation is well documented [1], recent studies also implicate SPR in the growth of brain tumors [2]. SPR is expressed in 9 out of 12 astrocytomas, 10 out of 10 glioblastomas, and the density of SPR correlates with the degree of malignancy, with glioblastomas expressing more receptors than astrocytomas [3]. At the molecular level, SPR stimulation in U373 MG glioblastoma cells increases mitogenesis, cell proliferation [4, 5], and interleukin-6 release [6]. SPR-dependent release

of interleukin-6 is noteworthy because it has been implicated in the progression of gliomas [7]. The evidence cited above suggests that SPR has an important role in the biology of glioblastomas.

The native expression of SPR in U373 MG cells, a human glioblastoma cell line, makes them a good model for studying the role of SPR signaling in glioblastoma growth. SPR activation in these cells stimulates phospholipase C, which hydrolyzes membrane phosphoinositides into two second messengers, inositol triphosphate and diacylglycerol, which stimulate intracellular calcium release and protein kinase C (PKC) activation, respectively [8, 9]. Recent studies implicate several molecules as having key roles in SPR signaling in U373 MG cells. They include mitogen-activated protein kinases ERK1/2 [4], mitogen-activated protein kinase p38 [10], transcription factor NF-κB [11], and epidermal growth factor receptor (EGFR) [12]. Of these molecules, ERK1/2 is activated by phosphorylation following stimulation of SPR in U373 MG cells, but the mechanism through which SP acts has not been investigated in detail [4]. A study by Castagliuolo et al. has shown that transactivated EGFR is a regulator of SP-induced ERK1/2 activation and subsequent cell proliferation [12].

The purpose of the present study is to better understand the relationship between SPR and glioblastoma growth by investigating the molecular events leading to ERK1/2 phosphorylation following SPR activation in U373 MG cells. We report that while SP-induced transactivation of EGFR has only a minor role in SP-dependent ERK1/2 phosphorylation, Src is a major mediator of SP-dependent ERK1/2 phosphorylation. We also report that Src mediates SP-dependent phosphorylation of PKCδ. Since rottlerin, a selective inhibitor of PKCδ, blocks SP-dependent ERK1/2 phosphorylation, our data suggest that PKCδ is involved in SPR signaling as well. Chelation of intracellular calcium by BAPTA/AM causes partial inhibition of SP-dependent ERK1/2 phosphorylation, indicating that calcium also contributes to SPR signaling in glioblastoma cells. Finally, we report that glioblastoma cell growth is decreased by both PP2 and rottlerin, suggesting that the cellular targets of these inhibitors are important regulators of growth in human glioblastomas.

Materials and methods

Materials

Substance P, human epidermal growth factor (EGF), phorbol-12-myristate-13-acetate (PMA), rottlerin, and IGEPAL CA-630 (equivalent detergent to NP-40) were from Sigma (St. Louis, MO, USA). Tyrphostin AG 1478, Bisindolylmaleimide I (GF109203X), 4-Amino-5-(4-chlorphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2), 4-Amino-7-phenylpyrazol[3,4-*d*]pyrimidine (PP3), and 1,2-*bis*-(*o*-Aminophenoxy)ethane-*N*, *N*, *N*′, *N*′-tetraacetic Acid Tetra-(acetoxymethyl) Ester (BAPTA/AM) were from Calbiochem (San Diego, CA, USA). The non-peptide SPR antagonist, CP99994-1, was provided by Dr. Paul Kadin (Pfizer Inc., Groton, CT, USA). Monoclonal antibody against EGFR, and horseradish peroxidase-conjugated goat-anti-mouse antibody were purchased from Upstate Biotechnology Inc. (Lake Placid, NY, USA). PhosphoPlus MAP kinase antibody kit was obtained from New England Biolabs (Beverly, MA, USA). Enhanced chemiluminescence reagent, SuperSignal West Pico, was obtained from Pierce (Rockford, IL, USA). [^{32}P]-*o*-phosphoric acid and protein A sepharose were from Amersham Biosciences (Piscataway, NJ, USA). Monoclonal antibody against tubulin, rabbit polyclonal antibodies against Src and PKCδ, and horseradish peroxidase-conjugated goat-anti-rabbit antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Monoclonal anti-phosphotyrosine antibody (PY20) was purchased from BD Transduction Laboratories (San Diego, CA, USA). The pcDNA3 expression vector, SH1(KD), containing the catalytic domain (a.a 250–536) of human Src with a kinase-inactivating mutation, K298M, and a carboxyl-terminal hemagluttinin tag [13] was kindly provided by Dr. Louis M. Luttrell (Duke University Medical Center, Durham, NC, USA). Small-interfering RNA (siRNA) directed against human EGFR and the Silencer siRNA transfection kit, including control RNA, were purchased from Ambion Inc. (Austin, TX, USA). Minimum essential medium (MEM), non-essential amino acids, sodium pyruvate, and fetal bovine serum were obtained from GibcoBRL Life Technologies (Grand Island, NY, USA). U373 MG, U87 MG, and DBTRG-05 MG cells were obtained from ATCC (Rockville, MD, USA) and were grown in MEM supplemented with 10% fetal bovine serum, 1 mM non-essential amino acids, 1 mM sodium pyruvate, and antibiotics in an atmosphere of 5% CO_2 at 37°C. The glioblastoma cell lines, D54 MG, D245 MG, D247 MG, and D502 MG, were provided by Dr. Darell D. Bigner (Duke University Medical Center), and were grown in zinc option medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum, 0.075% w/v sodium bicarbonate, and antibiotics in an atmosphere of 5% CO_2 at 37°C.

Solutions

Drug solutions were made as follows: EGF was 100 μg/ml in 10 mM Acetic acid and 0.1% BSA; SP was 1 mM in 20 mM Hepes, pH 8.0; Thapsigargin was 10 mM in 100% DMSO; AG 1478 was 20 mM in 100% DMSO; PP2 was 20 mM in 100% DMSO; PP3 was 20 mM in 100% DMSO; rottlerin was 20 mM in 100% DMSO; GF109203X was 10 mM in 100% MeOH; BAPTA/AM was 100 mM in 100% DMSO; and CP99,994-1 was 1 mM in H_2O.

Immunoblotting

U373 MG cells were seeded into 12-well tissue culture plates at a density of 2×10^5 cells/well and incubated in low-serum medium (0.5% FBS) for 12 h, followed by serum-free medium for 24 h at 37°C. The cells were then treated with drugs, as shown in the figure legends, and washed three times with ice-cold phosphate-buffered saline (PBS) prior to lysis in 0.1 ml of ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 5 mM $MgCl_2$; 0.1 mM Na_3VO_4; 10 mM $Na_4P_2O_7$; 1 mM EGTA; 10 mM NaF; 0.5% IGEPAL CA630; 1 mM benzamidine; 10 μg/ml leupeptin; 5 μg/ml aprotonin; 10 μg/ml soybean trypsin inhibitor; and 0.1 mM phenylmethylsulfonyl fluoride). Protein concentrations of cell lysates were measured by the Bradford Assay (Bio-Rad, Hercules, CA, USA). Twenty five micrograms of each lysate was combined with sample buffer (25 mM Tris–HCl, pH 6.8, 10% glycerol, 10% β-mercaptoethanol, and 0.01% bromophenol blue), the samples were resolved on 10% SDS polyacrylamide gel, and the proteins were then transferred to PVDF membranes (Immobilon-P, Millipore Corp, Bedford, MA, USA). Phospho-ERK1/2 was detected using the PhosphoPlus MAP kinase antibody kit according to the manufacturer's recommendations followed by enhanced chemiluminescence. Phosphotyrosine was detected with PY20 at a 1:1,000 dilution. Relative levels of phosphorylated proteins were quantitated by scanning densitometry performed on the images obtained by immunoblot (using ECL) and autoradiography using a GS-700 scanning densitometer and Quantity One software (BioRad).

Transfection of U373 MG cells with SH1(KD) and EGFR siRNA

For suppression of endogenous Src activity by transient expression of SH1(KD), U373 MG cells were seeded into 100-mm dishes at a density of 2×10^6 per dish, and allowed to attach overnight. The cells were then transfected with 5 μg of empty vector (pcDNA3) or SH1(KD) using LipofectAMINE Plus Reagent (Life Technologies, Inc.) according to the manufacturer's instructions. After incubating overnight, the transfected cells were seeded into a 12-well plate at a density of 2×10^5 cells /well. The cells were switched to serum-free medium 48 h post-transfection and serum-starved for an additional 24 h before assay. For suppression of EGFR expression with siRNA, U373 MG cells were plated into a 12-well plate at a density of 2×10^5 cells/well and allowed to attach overnight in growth medium. The cells were then transfected with 100 pmol/well of either control RNA or EGFR siRNA (Ambion Inc.), using siPORT Amine transfection reagent (Ambion Inc) according to the manufacturer's instructions. After incubating the cells with the siRNA overnight, fresh serum-free medium was added to the cells and they were assayed 48 h post-transfection by immunoblotting for EGFR and phosphotyrosine, as described above.

Phosphorylation of PKC in Intact U373 MG Cells

Substance P-dependent PKCδ phosphorylation was measured in intact U373 MG cells using a method similar to that described previously [14]. U373 MG cells were seeded into six-well dishes at a density of 5×10^5 cells/well and allowed to attach overnight. The cells were then washed three times with PBS, and incubated in 1 ml of phosphate-free medium containing 0.3 mCi of [^{32}P]-*o*-phosphoric acid for 2 h at 37°C. Following a 1-h pretreatment with the agents indicated in the figure legend, the radiolabeled cells were treated with or without 100 nM SP for 10 min. PKCδ was then immunoprecipitated from whole cell lysates with PKCδ antibody and detected by SDS-PAGE/autoradiography, as previously described except that the lysis buffer contained 0.5% IGEPAL CA-630 instead of 0.5% NP-40 [14, 15].

Cell proliferation assay

To measure cell proliferation, glioblastoma cells were seeded into six-well tissue culture dishes at a density of 1×10^5 cells/well and incubated under the growth conditions described above with the drugs indicated in the figure legend. Drug-containing growth medium was replaced every 24 h over a 72-h period. Cell proliferation at each time point was determined by treating cells with trypsin, suspending them, and counting them on a hemacytometer.

Results

EGFR has only a minor involvement in SP-dependent ERK1/2 phosphorylation in U373 MG cells

Transactivation of EGFR by SPR has previously been implicated as having a role in the mitogenic response of U373 MG cells to SP [12]. As shown in Fig. 1a, we find that blockade of EGFR activity with the specific EGFR kinase inhibitor, AG 1478, causes only partial inhibition of SP-dependent ERK1/2 phosphorylation (*upper panel, compare lanes 1 and 2 with lanes 5 and 6*), while the same treatment completely blocks ERK1/2 phosphorylation stimulated by EGF (*upper panel, compare lanes 3 and 4 with lanes 7 and 8*). Overall, AG 1478 inhibited SP-stimulated ERK1/2 phosphorylation by 26% ± 11 ($n=9$) whereas it inhibited EGFR-stimulated ERK1/2 phosphorylation by 93% ± 12 ($n=9$). Of note, we find that AG 1478 causes a small but consistent increase in the basal level of phosphorylated ERK1/2 (*upper panel, compare lanes 1 and 3 with lanes 5 and 7*); the underlying mechanism remains to be investigated. The role of

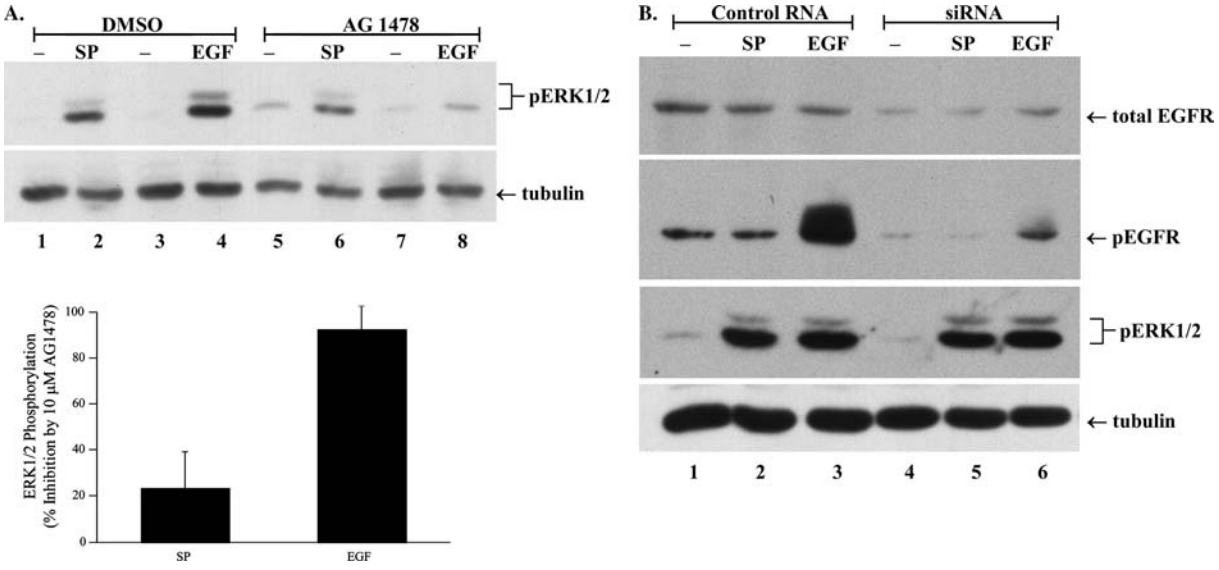

Fig. 1 Role of EGFR tyrosine kinase in SP-dependent ERK1/2 phosphorylation. **a** Serum-starved U373 MG cells were pretreated for 1 h at 37°C with vehicle (0.1% DMSO, *lanes 1–4*) or 10 μM AG 1478 (*lanes 5–8*), then stimulated for 10 min with 100 nM SP (*lanes 2 and 6*) or 100 ng/ml EGF (*lanes 4 and 8*). Whole cell lysates were immunoblotted for phospho-ERK1/2 (*upper panel*) or tubulin (*lower panel*), as described in "Materials and methods". *Bar graph*: Repetition of the experiment with 10 μM AG1478 yielded a mean percent inhibition of 26% ± 11 ($n = 9$) for SP-stimulated ERK1/2 phosphorylation and 93% ± 10 ($n = 9$) for EGF-stimulated ERK1/2 phosphorylation. **b** U373 MG cells were transfected with an inactive control RNA (*lanes 1–3*) or an siRNA directed against human EGFR (*lanes 4–6*), as described in "Materials and methods". At 48 h post-transfection, the cells were stimulated with 100 nM SP (*lanes 2 and 5*) or 100 ng/ml EGF (*lanes 3 and 6*) for 10 min at 37°C. Whole cell lysates were immunoblotted with anti-EGFR (*upper panel*), antiphosphotyrosine antibody (*second panel from top*), anti-phospho-ERK1/2 (*third panel from top*), or anti-tubulin as a control for protein loading on the gel (*bottom panel*)

EGFR in SP-stimulated ERK1/2 phosphorylation was further examined by suppressing the expression of native EGFR in U373 MG cells with siRNA directed against human EGFR. Figure 1b demonstrates a 70% reduction in EGFR expression following transfection with EGFR siRNA (*upper panel, compare lanes 1–3 with lanes 4–6*), and this reduction is reflected in the 78% decrease in tyrosine phosphorylation of EGFR following stimulation with EGF (*second panel from top, compare lane 3 with lane 6*). Decreased EGFR expression due to siRNA is also reflected in the decreased basal level of tyrosine phosphorylated EGFR (*second panel from top, compare lane 1 with lane 4*). While siRNA decreased the expression of EGFR, we did not find any decrease in SP- or EGF-dependent ERK1/2 phosphorylation (*third panel from top, compare lanes 2 and 3 with lanes 5 and 6*). This lack of inhibition despite the 70% reduction in EGFR expression suggests the presence of a large receptor reserve for EGFR in U373 MG glioblastoma cells. Based on our findings from the blockade of EGFR with AG 1478 and siRNA, we conclude that EGFR is not a major mediator of SP-dependent ERK1/2 phosphorylation in U373 MG cells.

PP2, a selective inhibitor of Src, blocks SP-dependent ERK1/2 phosphorylation in U373 MG cells

It has previously been reported that tyrphostin A25, a general tyrosine kinase inhibitor, blocks SP-stimulated DNA synthesis in U373 MG cells by approximately 80% [4], suggesting a role for tyrosine kinase activity in SP-dependent mitogenesis. To determine the identity of the tyrosine kinase mediating SPR signaling in U373 MG cells, we examined the non-receptor tyrosine kinase, Src, because it is known to mediate the function of several GPCRs [16], including recombinant SPR in KNRK cells [17]. As shown in Fig. 2a, SP-dependent ERK1/2 phosphorylation in U373 MG cells is decreased in a concentration-dependent manner by the Src kinase inhibitor, PP2 (Fig. 2a. *upper panel*). In contrast, stimulation of ERK1/2 phosphorylation by EGF was not significantly affected by PP2 (Fig. 2a. *upper panel, lanes 11 and 12*). Additional experiments using 20 μM PP2, a dose causing maximal inhibition of ERK1/2 phosphorylation, yielded a mean of 40% ± 16 (*Bar graph*; $n = 8$) for inhibition of SP-stimulated ERK1/2 phosphorylation. In contrast, SP-dependent ERK1/2 phosphorylation was not inhibited by PP3 (data not shown), a structural analog of PP2 that does not inhibit Src [18]. This result confirms that the reduction in SP-dependent ERK1/2 phosphorylation by PP2 is due to its inhibition of Src.

To further establish the role of Src in SP-dependent ERK1/2 phosphorylation, we blocked Src activity in U373 MG cells by transiently expressing a kinase-dead Src mutant, SH1 (KD). As shown in Fig. 2b, transfection of U373 MG cells produced robust expression of SH1(KD) (*lane 3*). In Fig. 2c, SP-dependent ERK1/2 phosphorylation is shown to be inhibited by 70% in the presence of SH1 (KD) (*upper panel, compare lane 2 with*

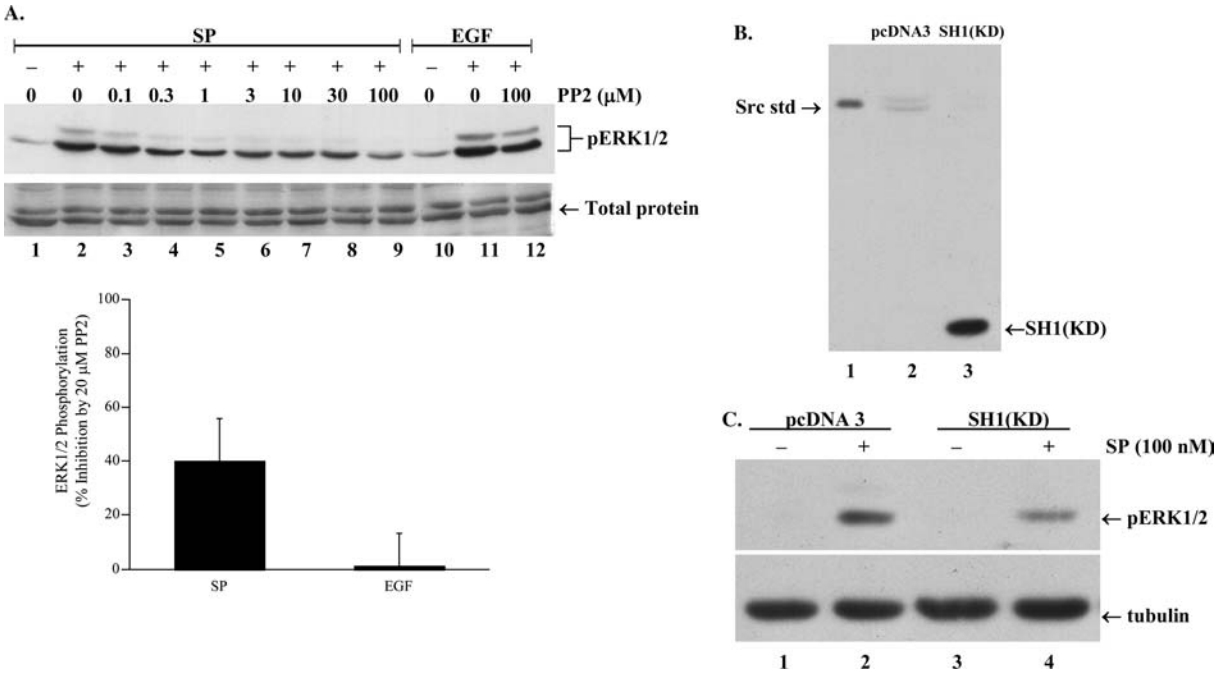

Fig. 2 Role of Src in SP-dependent ERK1/2 phosphorylation. **a** Serum-starved U373 MG cells were pretreated for 1 h at 37°C with a range of PP2 concentrations (*lanes 3–9, and 12*,) or with vehicle (0.1% DMSO, *lanes 1, 2, 10, and 11*). Cells were then stimulated with 100 nM SP (*lanes 2–9*) or 100 ng/ml EGF (*lanes 11 and 12*) for 10 min at 37°C. Whole cell lysates were immunoblotted for phospho-ERK1/2 as described in "Materials and methods" (*upper panel*). Equal protein loading on the gel is demonstrated by Coomassie staining of total protein (*lower panel*). *Bar graph*: Repetition of the experiment with 20 μM PP2 yielded a mean percent inhibition of 40% ± 16 ($n = 8$) for SP-stimulated ERK1/2 phosphorylation, and no significant inhibition of EGFR-stimulated ERK1/2 phosphorylation ($n = 8$). **b** U373 MG cells were transfected with pcDNA3 (*lane 2*) or SH1 (KD) (*lane 3*), as described in "Materials and methods". Immunoblotting of whole cell lysates for Src shows purified full-length c-Src standard (*lane 1*), endogenous Src *(lane 2*), and SH1(KD) expression (*lane 3*). *C* U373 MG cells were transfected with pcDNA3 (*lanes 1 and 2*) or SH1(KD) (lane 3 and 4), as described in "Materials and methods". At 72 h post-transfection, the cells were stimulated with no addition (*lanes 1 and 3*) or 100 nM SP (*lanes 2 and 4*) for 10 min at 37°C. Whole cell lysates were immunoblotted for phospho-ERK1/2 (*upper panel*) or tubulin (*lower panel*) as described in "Materials and methods"

lane 4). Thus, a decrease in SP-dependent ERK1/2 phosphorylation following inhibition of Src activity by both pharmacological and DNA-based approaches supports the conclusion that Src is a mediator of SP-dependent ERK1/2 phosphorylation in U373 MG cells.

PP2 blocks SP-dependent PKC phosphorylation in U373 MG cells

Src-dependent PKCδ phosphorylation has been reported following stimulation of rat acinar cells with either SP [19] or cholecystokinin [20]. Therefore, we next determined whether SP stimulates PKCδ phosphorylation in glioblastoma cells, and whether Src mediates this effect. U373 MG cells were metabolically labeled with [^{32}P]-orthophosphate, then stimulated with substance P in the absence or presence of the Src inhibitor, PP2, the inactive PP2 analog, PP3, or the nonpeptide SPR antagonist, CP99,994-1. PKCδ was subsequently immunoprecipitated from cell lysates. As shown in Fig. 3a, stimulation with SP increases phosphorylation of PKCδ (*Upper panel, compare lane 2 with lane 1*). Scanning densitometry indicated that the increase was 140% of the basal level. This increase in PKCδ phosphorylation was almost completely blocked by PP2 (Fig. 3a, *Upper panel, lane 3*) and by the SPR antagonist, CP99,994-1 (*Upper panel, lane 4*), reducing it to < 110% of the basal in both cases. PKCδ phosphorylation was not affected by PP3 (*Upper panel, lane 5*). These findings indicate that SP-dependent phosphorylation of PKCδ is mediated by Src.

There have been reports of PKCδ involvement in the stimulation of ERK1/2 phosphorylation by endothelin receptor in rat myometrial cells [21] or bradykinin receptor in breast epithelial cells [22]. To determine whether PKCδ has a role in SP-dependent ERK1/2 phosphorylation, we stimulated U373 MG cells with SP in the absence or presence of rottlerin, a PKC inhibitor having selectivity for PKCδ. As shown in Fig. 3b, rottlerin inhibits SP-induced ERK1/2 phosphorylation by 69% (*Upper panel, compare lane 7 with lane 3*) and PMA-stimulated ERK1/2 phosphorylation by 57% (*Upper panel, compare lane 6 with lane 2*). Of note, both rottlerin and GF109203X cause a small decrease in the basal phosphorylation of ERK1/2 (*Upper panel, compare lanes 5 and 9 with lane 1*). In contrast to its effect on SPR signaling, rottlerin did not decrease EGF-dependent ERK1/2 phosphorylation (*Upper panel, compare lane 8 with lane 4*), suggesting that the inhibitory effect of rottlerin is specific to SPR signaling.

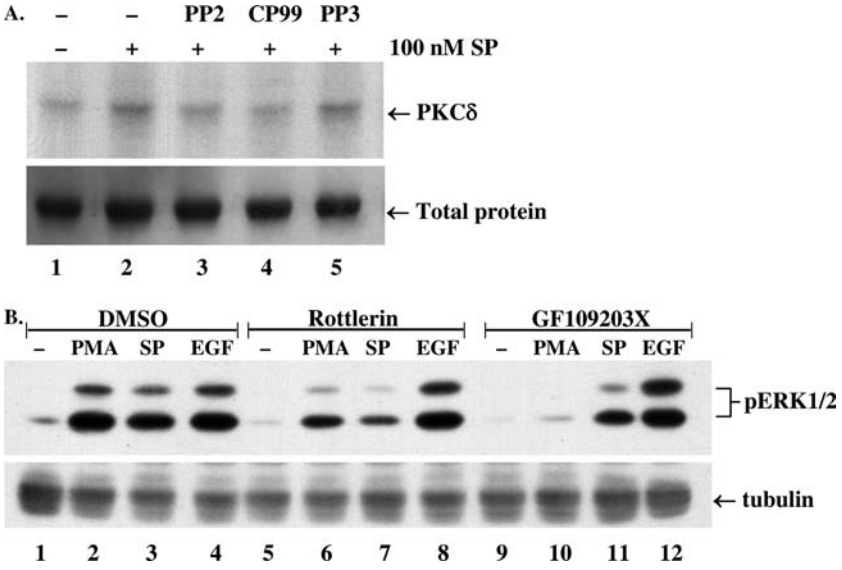

Fig. 3 Role of PKCδ in SP-dependent ERK1/2 phosphorylation. **a** U373 MG cells were incubated for 1 h at 37°C with 0.3 mCi [^{32}P]-*o*-phosphoric acid in phosphate-free medium as described in "Materials and methods". The cells were treated with 0.1% DMSO (*lanes 1 and 2*), 20 μM PP2 (*lane 3*), 1 μM CP99,994-1 (*lane 4*), or 20 μM PP3 (*lane 5*) for 1 h, then stimulated with 100 nM SP (*lanes 2-5*) for 1 min at 37°C. Cell lysates were precleared with protein A sepharose, then immunoprecipitated with antibody specific for PKCδ. Immunoprecipitated phospho-PKCδ is shown by SDS-PAGE/autoradiography (*upper panel*). Equal loading of lanes on the gel is demonstrated by Coomassie staining of total protein (*lower panel*). **b** Serum-starved U373 MG cells were pretreated for 1 h at 37°C with 0.1% DMSO (*lanes 1–4*), 10 μM rottlerin, (*lanes 5–8*), or 10 μM GF109203X (*lanes 9-12*), then stimulated for 10 min with 100 nM PMA (*lanes 2, 6 and 10*), 100 nM SP (*lanes 3, 7 and 11*), or 100 ng/ml EGF (*lanes 4, 8, and 12*). Whole cell lysates were immunoblotted for phospho-ERK1/2 (*upper panel*) or tubulin (*lower panel*) as described in "Materials and methods"

BAPTA/AM, a chelator of intracellular calcium, blocks SP-dependent ERK1/2 phosphorylation

Since SPR is coupled to phosphotidylinositol hydrolysis, resulting in the release of inositol triphosphates and intracellular calcium [8, 9], we determined whether calcium is involved in SP-dependent ERK1/2 phosphorylation. As shown in Fig. 4, BAPTA/AM, a calcium chelating agent, partially inhibits SP-stimulated ERK1/2 phosphorylation (*upper panel, compare lane 4 with lane 2*), indicating that calcium is involved in SP-dependent ERK1/2 phosphorylation. In contrast, BAPTA/AM has very little effect on EGF-stimulated ERK1/2 phosphorylation (*upper panel, compare lane 12 with lane 10*). As shown in the bar graph, the mean inhibition by BAPTA/AM was 46% ± 28 ($n = 5$) for SP-stimulated ERK1/2 phosphorylation and 11% ± 5 ($n = 5$) for EGF-stimulated ERK1/2 phosphorylation. Partial blockade of SP-dependent ERK1/2 phosphorylation by BAPTA/AM was not due to incomplete removal of intracellular calcium, because BAPTA/AM completely inhibited the stimulation of ERK1/2 phosphorylation by thapsigargin (*upper panel, compare lane 8 with lane 6*), a compound that releases intracellular calcium by inhibiting endoplasmic reticular Ca^{2+}-ATPase [23]. We conclude that calcium is a mediator of SP-dependent ERK1/2 phosphorylation in U373 MG glioblastoma cells.

PP2 and rottlerin inhibit the growth of glioblastoma cells

Having established that PP2 and rottlerin both inhibit SP-dependent ERK1/2 phosphorylation, we examined the effects of these two inhibitors on glioblastoma growth. Figure 5a shows the effect of PP2 on the growth of three glioblastoma cell lines incubated for 3 days in normal growth medium containing 20 μM PP2, vehicle (0.1% DMSO), or no addition (control). We found that DMSO has no effect on cell growth (data not shown). After 3 days, PP2 decreased the growth of all three cell lines to less than half that of the control cells, confirming that Src has a role in glioblastoma growth. Figure 5b shows the effect of rottlerin on the growth of six different human glioblastoma cell lines incubated for 3 days in growth medium containing 10 μM rottlerin or 0.1% DMSO. As can be seen, the growth of all six cell lines was completely suppressed by rottlerin. This result is consistent with a previous report in which rottlerin inhibited the growth of two other glioblastoma cell lines, U138 MG and T98G [24].

Discussion

Substance P Receptor has been implicated in the growth and malignancy of glioblastoma cells [3, 4]. The present

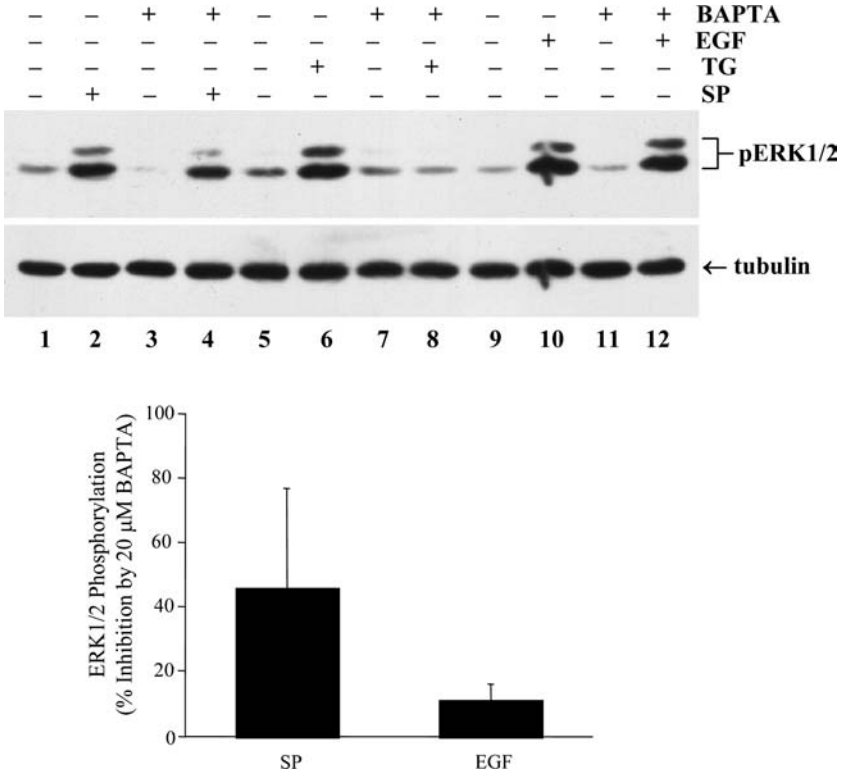

Fig. 4 Role of Calcium in SP-dependent ERK1/2 phosphorylation. Serum-starved U373 MG cells were pretreated for 1 h at 37°C with vehicle (0.1% DMSO, *lanes 1, 2, 5, 6, 9, and 10*) or 20 μM BAPTA (*lanes 3, 4, 7, 8, 11, and 12*), then stimulated for 10 min with 100 nM SP (*lanes 2 and 4*), 2 μM thapsigargin (*lanes 6 and 8*), or 100 ng/ml EGF (*lanes 10 and 12*). Whole cell lysates were then immunoblotted for phospho-ERK1/2 (*upper panel*) or tubulin (*lower panel*), as described in "Materials and methods". *Bar graph* : Repetition of the experiment with 20 μM BAPTA yielded a mean percent inhibition of 46% ± 28 ($n = 5$) for SP-stimulated ERK1/2 phosphorylation and 11% ± 5 ($n = 5$) for EGF-stimulated ERK1/2 phosphorylation

study examines the mechanism by which SPR activation leads to the phosphorylation of ERK1/2, a kinase involved in growth control. It has been previously reported that ERK1/2 is phosphorylated following transactivation of EGFR by SPR [12]. Our results indicate that transactivation of EGFR by SPR is not the predominant mechanism acting in SP-dependent ERK1/2 phosphorylation. This conclusion is based on two observations; first, SP-dependent ERK1/2 phosphorylation is only partially blocked by the EGFR kinase inhibitor, AG 1478, and secondly, suppression of EGFR expression by siRNA has no inhibitory effect on SP-dependent ERK1/2 phosphorylation. While transactivation of EGFR by SPR has a role in SP-dependent ERK1/2 phosphorylation, our data show Src to be a key mediator of SP-dependent ERK1/2 phosphorylation in U373 MG glioblastoma cells. Blockade of Src activity by either PP2 or expression of a kinase-dead Src mutant, SH1(KD), causes inhibition of SPR-mediated ERK1/2 phosphorylation. Further, we show that PP2 blocks SP-dependent phosphorylation of PKCδ, suggesting that PKCδ may also be a component of this pathway. Such a role for PKCδ is consistent with our demonstration that the subtype-selective PKC inhibitor, rottlerin, blocks SP-dependent ERK1/2 phosphorylation. Finally, we demonstrate that inhibition of two of the cellular kinases activated by SPR, namely Src and PKCδ, inhibits the growth of several glioblastoma cell lines.

Our demonstration of Src as a mediator of SPR-dependent ERK1/2 phosphorylation in U373 MG cells identifies it as a potential target for the design of drugs to block the proliferation of human glioblastoma cells. Considered within this context, it is noteworthy that Src has already been shown to play a key role in several other types of cancer including colon, breast, and lung [25]. Evidence for the involvement of Src in colon cancer is especially strong because elevated Src activity (2- to 40-fold) has been found by several investigators, and blockade of Src decreases the tumorigenicity of colon carcinoma cells [26]. More recently, an activating mutation in Src was revealed in a subset of advanced human colon cancers [27]. Based on these observations, there currently is renewed interest in the evaluation of Src inhibitors as anti-cancer agents [28, 29]. With respect to glioblastomas, the role of Src has not previously been investigated in detail, perhaps because of an earlier study that found no elevation of Src activity in glioblastomas [30]. While Src activity in glioblastomas may not be elevated as much as other types of cancers, the potential for Src to act in the control of glioblastoma cell growth is demonstrated by our finding that inhibition of Src blocks both SPR signaling along the ERK1/2 phosphorylation pathway and normal growth of glioblastoma cell lines.

Our data also suggest that PKCδ may be a mediator of SP-dependent ERK1/2 phosphorylation in glioblastoma cells. This is consistent with the report of SP-dependent PKCδ phosphorylation through Src in rat acinar cells [19]. While a number of studies connect PKCδ activation with induction of apoptosis [31–34], PKCδ is also reported to mediate the transformation of NIH 3T3 cells following activation of insulin-like growth factor I receptor [35]. It has been suggested that the variety of effects reported for PKCδ is the result of

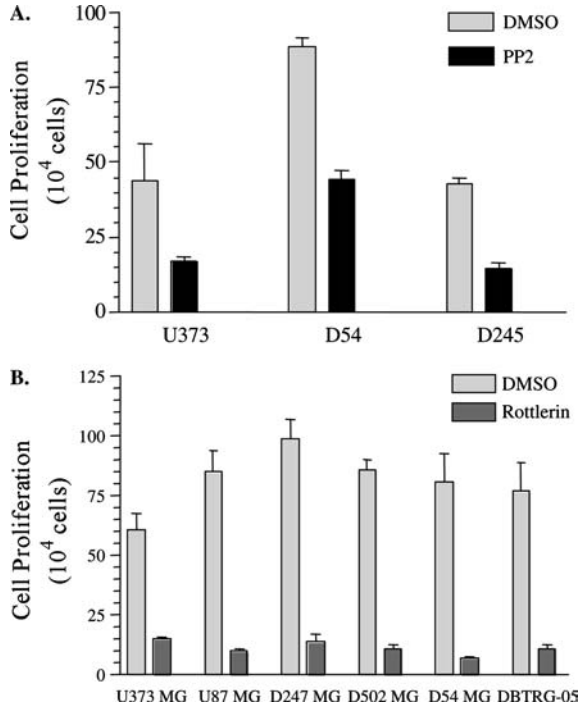

Fig. 5 Roles of Src and EGFR kinase in normal growth of glioblastoma cells. **a** Three different glioblastoma cell lines were seeded into six-well tissue culture dishes at a density of 1×10^5 cells/well in MEM medium containing 10% FBS with 0.1% DMSO (*gray bars*) or 20 μM PP2 (*black bars*) and cells were counted at 24, 48, and 72 h after the addition of drugs as described in "Materials and methods"; cell counts at 72 h are shown. **b** Six different glioblastoma cells were seeded into six-well tissue culture dishes at a density of 1×10^5 cells/well in normal growth medium containing 10% FBS with either 0.1% DMSO (*gray bars*) or 10 μM rottlerin (*black bars*). The cells were counted in duplicate after 72 h

differential phosphorylation of specific tyrosine residues on PKCδ by different stimuli [36]. Such a regulatory mechanism would allow PKCδ to induce either apoptosis or cell proliferation depending on the stimulus or the cell type [36]. Consistent with our results, several recent studies report that PKCδ promotes proliferative signals like ERK1/2 phosphorylation and increased c-Fos expression following activation of Gq-coupled receptors. Examples include bradykinin in epithelial breast cells [22], ATP acting through a purinergic receptor in vascular smooth muscle cells [37], and endothelin-1 in rat myometrial cells [21]. Future investigations will determine more precisely the role of PKCδ in the proliferation of human glioblastomas.

Given that there are currently no effective drugs available for the treatment of glioblastomas, our finding that PP2 inhibits growth in glioblastoma cells is significant and reinforces the idea that Src is a target for drug development. We also report growth inhibition by rottlerin in six glioblastoma cell lines, extending the findings of Parmer et al.[24], who reported the inhibitory effect of rottlerin in two other glioblastoma cell lines. At present, the mechanism of this inhibitory effect is unknown since rottlerin has been reported to inhibit both PKCδ [38] and Ca^{2+}/calmodulin kinase III (also called eukaryotic elongation factor 2 kinase) [24]. Regardless of the mechanism, the fact that glioblastoma growth is effectively inhibited by rottlerin suggests that it represents a good starting point for the development of drugs to treat glioblastomas.

Our study also points out a striking difference between SPR and EGFR in the mechanism by which these receptors increase the phosphorylation of ERK1/2. While SPR-mediated ERK1/2 phosphorylation involves calcium, PKCδ, and Src, none of these molecules mediate EGFR-dependent ERK1/2 phosphorylation. These differences between SPR, a member of the GPCR superfamily, and EGFR, a member of the receptor tyrosine kinase family, are likely to have importance for the design of agents to inhibit glioblastoma growth. Blockade of EGFR kinase in combination with other anti-tumor agents has been shown to improve inhibition of glioblastoma growth, extending the life span of tumor-bearing mice [39, 40]. However, inhibition of signaling pathways involving GPCRs in conjunction with EGFR inhibitors has not been attempted and may yield better results.

In summary, the present study provides evidence that in human glioblastoma cells, activation of SPR is linked to ERK1/2 phosphorylation through a mechanism involving Src and PKCδ. Further, inhibition of Src or PKCδ inhibits the growth of human glioblastoma cells, suggesting that Src is a potential target for the development of therapeutic agents designed to inhibit the growth of human glioblastomas.

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