Bombesin and Substance P Analogues Differentially Regulate G-protein Coupling to the Bombesin Receptor

DIRECT EVIDENCE FOR BIASED AGONISM*

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Substance P analogues including [D-Arg¹,D-Phe⁵,D-Trp⁷,⁹,Leu¹¹]substance P (SpD) act as "broad spectrum neuropeptide antagonists" and are potential anticancer agents that inhibit the growth of small cell lung cancer cells in vitro and in vivo. However, their mechanism of action is controversial and not fully understood. Although these compounds block bombesin-induced mitogenesis and signal transduction, they also have agonist activity. The mechanism underlying this agonist activity was examined. SpD binds to the ligand-binding site of the bombesin/gastrin-releasing peptide receptor and blocks the bombesin-stimulated increase in [Ca²⁺]i within the same concentration range that causes sustained activation of c-Jun N-terminal kinase and extracellular signal-regulated protein kinase (ERK). The activation of c-Jun N-terminal kinase by SpD and bombesin is blocked by dominant negative inhibition of Gα12. The ERK activation by SpD is pertussis toxin-sensitive in contrast to ERK activation by bombesin, which is pertussis toxin-insensitive but dependent on epidermal growth factor receptor phosphorylation. SpD does not simply act as a partial agonist but differentially modulates the activation of the G-proteins Gα12, Gαi, and Gγ, compared with bombesin. This unique ability allows the bombesin receptor to couple to Gαi and at the same time block receptor activation of Gγ. Our results provide direct evidence that SpD is acting as a "biased agonist" and that this has physiological relevance in small cell lung cancer cells. This validation of the concept of biased agonism has important implications in the development of novel pharmacological agents to dissect receptor-mediated signal transduction and of highly selective drugs to treat human disease.

Neuropeptides have been implicated in the pathogenesis of a number of human disease states including inflammatory disease, cardiovascular disease, and cancer (1). Neuropeptides including bombesin are autocrine growth factors for a number of cancers including breast, prostate, and small cell lung cancer (SCLC) (2–5). In particular, neuropeptides and their receptors are the principle driving force behind one of the most clinically aggressive cancers, SCLC. SCLC cells sustain their growth in vitro as part of a result of multiple autocrine and paracrine loops involving calcium-mobilizing neuropeptides (6, 7). SCLC provides a paradigm for the investigation of neuropeptide-mediated growth. Modulating neuropeptide-induced signal transduction may therefore have important implications in the treatment of a number of human diseases.

Neuropeptides are a structurally diverse group of hormones and neurotransmitters that bind to a related subfamily of G-protein-coupled receptors. Predominately, these receptors couple to Gq to elicit phospholipase C-β activation and subsequent production of diacylglycerol and phosphatidylinositol 1,4,5-trisphosphate leading to protein kinase C activation and Ca²⁺ release (8–10). These receptors also couple to Gα12 to elicit c-Jun N-terminal kinase (JNK) activation and Rho-dependent activation of stress fiber formation via tyrosine phosphorylation of a number of tyrosine kinases including FAK, paxillin, and p130Cas (11–14). Bombesin and other neuropeptides have also been shown to activate the ERK pathway leading to the stimulation of immediate early genes and proliferation (10, 15, 16).

Analogs of substance P [D-Arg¹,D-Phe⁵,D-Trp⁷,⁹,Leu¹¹]-substance P (SpD) and [Arg⁶,D-Trp⁷,⁹,NmePhe⁸]substance P (6–11) can inhibit neuropeptide-stimulated Ca²⁺ mobilization, tyrosine phosphorylation, and ERK activation (17–19). Crucially, SpD and [Arg⁶,D-Trp⁷,⁹,NmePhe⁸]substance P inhibit SCLC cell growth in vivo and in vitro (7, 20) and stimulate SCLC cell apoptosis (21, 22). Substance P analogues are about to enter phase II clinical investigation for the treatment of SCLC and could provide a novel form of therapy for other neuroendocrine tumors in addition to SCLC (23). Hence understanding the mechanism of action of this class of compound is attracting considerable interest and is critical for future drug development.

Substance P analogues were characterized originally as "broad spectrum neuropeptide antagonists" (7, 17–19). However, the precise mechanism of action of these compounds seems more complex and remains unclear and contentious (23). Studies in Swiss 3T3 cells suggested that substance P analogues competitively inhibit the binding of neuropeptides to...
their receptors, accounting for the inhibition of neuropeptide-stimulated signal transduction (17, 18). Substance P analogues were thought to inhibit SCLC cell growth by competitively inhibiting the mitogenic effects of autocrine neuropeptides (17–20). However, the SCLC cell growth inhibitory and proapoptotic activities of substance P analogues are not reversed by supramaximal saturating concentrations of neuropeptides (21). Furthermore, substance P analogues themselves activate JNK and potentiate bradykinin-induced edema formation in rabbit skin (21, 23, 24). Further studies have shown that although SpD irreversibly blocks bombesin-induced phospholipase C-β activation and mitogenesis and reversibly inhibits ERK activation by bombesin (25), SpD augments Raf-1 and ERK activation by high concentrations of bombesin (25). This suggests that the GRP receptor may still be capable of signaling even when bombesin-induced phospholipase C activation is fully blocked. Thus in addition to its well described antagonist activity, SpD also has agonist activity. The ability of a receptor to have more than one active state and interact with multiple G-proteins to produce cellular responses has been described in a variety of receptor systems and has been termed “agonist-receptor trafficking” (26, 27). Jarpe et al. (24) hypothesized that SpD may act as an agonist at GRP receptors, activating the G₁₂ family of guanine-nucleotide binding proteins while blocking signal transduction via G₄ and proposed a novel pharmacological term, “biased agonism,” to describe this. Agents acting by the mechanism of biased agonism that selectively activate G-proteins would have enormous pharmacological and clinical importance and could become valuable tools in the dissection of signal transduction pathways downstream of receptors. However, the hypothesis of biased agonism was challenged by Sinnett-Smith et al. (28), who concluded that substance P analogues acted primarily as antagonists of neuropeptide receptors, blocking signal transduction via both G₁₂ and G₄ and that any agonist activity was caused by partial agonism.

The experiments presented here were designed to examine the mechanism underlying the agonist activity of SpD and to investigate specifically the validity of the biased agonist hypothesis.

**EXPERIMENTAL PROCEDURES**

**Materials**—Rat-1a cells and rat-1a cells stably expressing the mouse bombesin/GRP receptor (BORG-15) were established by the Imperial Cancer Research Fund (London). COS-7 cells and NCI-H358 SCLC cells were purchased from the American Type Tissue Culture Collection (Manassas, VA). Native Balb 3T3 cells and Balb 3T3 cells expressing the mutant bombesin receptors (5ET4 and R288H) were supplied by Dr. J. F. Battey (National Institutes of Health, Rockville, Maryland). Dominant negative G-protein α subunit cDNAs G₁₂ (G228A) and G₁₃ (G225A) in the pCIS transfection vector were supplied kindly by Dr. Stefan Offermans (Freie Universität Berlin, Germany). RPMI 1640 medium, Dulbecco’s modified Eagle’s medium (DMEM), bombesin, anti-phospho-ERK₁/₂ monoclonal antibody, myelin basic protein, and SpD were from Sigma; [γ-³²P]ATP (3000 Ci/mmol), [γ-³²P]ATP (3000 Ci/ mmol), and [³²P]-GRP (2000 Ci/mmol) were from Amersham Pharmacia Biotech; anti-ERK₁/₂, anti-JNK₁/₂, anti-phospho-JNK₁/₂, anti-α1, anti-phosphorylated α1, and glutathione S-transferase-c-Jun (79) substrate. Phosphorylated c-Jun was identified from autoradiographs of Coomassie Blue-stained SDS-PAGE gels and quantified by phosphorimaging. HA-JNK was precipitated from transiently transfected COS-7 cells using 2 μg of anti-HA antibody coupled to Sepharose (Santa Cruz Biotechnology). An aliquot of lyase was retained and boiled in Laemmli SDS-PAGE loading buffer for future Western analysis. Immune complexes from cell lysates containing 400 μg of protein were washed three times at 4 °C in 20 μM HEPES, pH 7.4, containing 50 μM NaCl, 2.5 mM MgCl₂, and 0.1 mM EDTA, 0.5% Triton X-100, 20 μM β-glycerophosphate, and 0.5 μM sodium orthovanadate and protease inhibitors (the protease inhibitor mixture was from Roche Molecular Biochemicals and prepared as per manufacturer instructions). Immune complexes from cell lysates containing 400 μg of protein were washed three times at 4 °C in 20 μM HEPES, pH 7.4, containing 50 μM NaCl, 2.5 mM MgCl₂, and 0.1 mM EDTA, 0.5% Triton X-100, 20 μM β-glycerophosphate, and 0.5 μM sodium orthovanadate. Kinase activity was estimated in 25 μl of kinase buffer containing 100 μM ATP, 1 μCi of [γ-³²P]ATP (3000 Ci/mmol), and 10 μg of myelin basic protein. The reaction was carried out for 20 min at 30 °C and terminated by spotting the supernatant onto P81 phosphocellulose paper. The papers were washed three times in 0.5% (v/v) phosphoric acid and dried briefly in aceton. The results are expressed as specific disintegrations per minute over background.

**Measurement of JNK Activity**—Cell lysates were generated as described for ERK₁/₂. After immunoprecipitation of JNK₁, from whole-cell lysates, kinase activity was carried out as described (21) using 20 μM ATP, 1 μCi of [³²P]ATP (3000 Ci/mmol), and 1 μg of glutathione S-transferase-c-Jun (79) substrate. Phosphorylated c-Jun was identified from autoradiographs of Coomassie Blue-stained SDS-PAGE gels and quantified by phosphorimaging. HA-JNK was precipitated from transiently transfected COS-7 cells using 2 μg of anti-HA antibody coupled to Sepharose (Santa Cruz Biotechnology). JNK activity was determined as described above.

**Western Blotting**—Whole-cell lysates were normalized for protein concentration (typically 1.0–2.0 mg/ml) and denatured by boiling (5 min) in SDS-PAGE loading buffer. 20 μl of lysate/lane was resolved on 12% SDS-PAGE gels and electroblotted onto nitrocellulose membranes. The membranes were blocked in 5% nonfat milk in PBS containing 0.05% Tween 20, ERK₁/₂, and JNK₁/₂ phosphorylation was determined using 1:1000 dilution of the primary antibody followed by the appropriate hors eradish peroxidase-labeled goat IgG (DAKO) diluted 1:5000. Bands were visualized using enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech). Anti-phospho-ERK₁/₂ monoclonal antibody, anti-phospho-JNK₁/₂, anti-α1, anti-phosphorylated α1, and glutathione S-transferase-c-Jun (79) substrate. Phosphorylated c-Jun was identified from autoradiographs of Coomassie Blue-stained SDS-PAGE gels and quantified by phosphorimaging. HA-JNK was precipitated from transiently transfected COS-7 cells using 2 μg of anti-HA antibody coupled to Sepharose (Santa Cruz Biotechnology). JNK activity was determined as described above.
**RESULTS**

**SpD Blocks Bombesin-induced \( \text{Ca}^{2+} \) Mobilization**—The mechanism underlying the agonist activity of SpD is unclear and controversial. SpD can bind to multiple characterized and uncharacterized G-protein-linked receptors. To define precisely the mechanism underlying the agonist activity of SpD, we used a rat-1a fibroblast model system (into which the bombesin receptor is transfected) to mediate SpD activity. Bombesin and other \( \text{Ca}^{2+} \)-mobilizing neuropeptides (vasopressin, neurotensin, bradykinin, and gastrin), fail to mobilize intracellular \( \text{Ca}^{2+} \) in native rat-1a fibroblasts, suggesting the absence of these receptors in this cell line (Fig. 1A). In rat-1a cells stably expressing the bombesin/GRP receptor (BOR-15), bombesin induces a marked and rapid increase in [\( \text{Ca}^{2+} \)], which was blocked by SpD (Fig. 1B). In addition, SpD at concentrations as high as 100 nM failed to mobilize intracellular calcium in rat-1a or BOR-15 cells (Fig. 1C). In BOR-15 cells, bombesin induced a concentration-dependent increase in [\( \text{Ca}^{2+} \)], with EC\(_{50} = 1.3 \pm 0.2 \text{nM}\). These results are similar to those seen for bombesin stimulation of [\( \text{Ca}^{2+} \)], in Swiss 3T3 cells (7, 17). SpD (30 nM) shifted the bombesin concentration response curve to the right (Fig. 1D, inset) with a [\( \text{K} \)] for the inhibition of bombesin-stimulated \( \text{Ca}^{2+} \) flux of 6.4 ± 0.7 \text{nM}. These results are similar to those seen for SpD inhibition of both bombesin-induced stimulation of [\( \text{Ca}^{2+} \)], and DNA synthesis in Swiss 3T3 cells\(^2\) (7, 17) and for the inhibition of [\( ^{125} \text{I} \)]-GRP receptor binding (Fig. 5).

**SpD Stimulates ERK at Concentrations That Inhibit Bombesin-stimulated \( \text{Ca}^{2+} \) Release**—The addition of SpD for 10 min causes a marked concentration-dependent increase in ERK activity in quiescent rat-1a fibroblasts expressing the bombesin receptor (BOR-15 cells), in contrast to the untransfected rat-1a cells (Fig. 2). In BOR-15 cells, bombesin stimulation of ERK activity was seen in the \text{nM} range, maximal at 30 nM (EC\(_{50} = 5.9 \pm 1.8 \text{nM}\), n = 4). SpD-induced stimulation of ERK activity in BOR-15 cells was evident at 3 \text{\mu M}, maximal at 10 \text{\mu M} (EC\(_{50} = 4.19 \pm 0.6 \text{\mu M}\), n = 3) (Fig. 2). To ensure that this effect was not a result of clonal selection, cells were transiently transfected with the mouse bombesin receptor, and similar results were obtained (results not shown). The stimulation of ERK occurred within the same concentration range at which SpD inhibits bombesin-stimulated \( \text{Ca}^{2+} \) release.

The time course of ERK activation by SpD is different from that of bombesin (Fig. 3). ERK\(_{1/2}\) phosphorylation as an indication of ERK activity was assessed by immunoblotting with a phospho-specific monoclonal antibody to ERK\(_{1/2}\) and showed quantitatively similar results to that of the immunoprecipitation kinase assay (data not shown). SpD-induced ERK\(_{1/2}\) phosphorylation was evident at 5 min, reached a maximum at 10 min, and persisted for over 60 min. The time course of ERK\(_{1/2}\) activation by SpD was slower in onset and more sustained in comparison with that of bombesin, which returned to control levels by 30 min (Fig. 3). This suggests that SpD and bombesin differentially regulate signal transduction pathways leading to ERK activation.

**SpD Acts at the Ligand-binding Site of the Bombesin Receptor to Induce ERK Activation**—SpD- and bombesin-induced ERK activity in BOR-15 cells was inhibited by two specific GRP/bombesin receptor antagonists, \([\text{Leu}^{15}(\text{CH}_3\text{NH})-\text{Leu}^{14}]\) bombesin and RC-3095 (32). Pretreatment of BOR-15 cells for 30 min with \([\text{Leu}^{15}(\text{CH}_3\text{NH})-\text{Leu}^{14}]\) bombesin (30 nM) or 1 \text{nM} RC-3095 (1 \text{nM}) completely blocked the ERK activity stimulated by either bombesin (30 nM) or SpD (30 nM, Fig. 4A). These results suggest that SpD-induced ERK activation is GRP receptor-dependent and occurs in the absence of a calcium signal.

Bombesin did not stimulate an increase in [\( \text{Ca}^{2+} \)], in Balb 3T3 cells. In Balb 3T3 cells expressing the wild-type GRP receptor (5ET4), 30 nM bombesin stimulated a maximal increase in [\( \text{Ca}^{2+} \)], of 375 nM. However, in Balb 3T3 cells expressing the mutated receptor (R288H), 30 nM bombesin did not increase [\( \text{Ca}^{2+} \)], with 100 nM bombesin increasing [\( \text{Ca}^{2+} \)], by 47 nM (results not shown). This is consistent with previously reported data showing that Arg-288 in the bombesin/GRP receptor is...

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\(^2\) A. C. MacKinnon and T. Sethi, unpublished observations.
critical for high affinity bombesin binding and that the R288H mutation does not affect GRP receptor expression or conformation and otherwise the R288H mutant GRP-receptor is fully functional (33, 34). SET4 or R288H cells were stimulated with bombesin (3 nM) or SpD (0.3–30 μM) for 10 min. Bombesin (3 nM) caused a 2.6-fold stimulation of ERK in 5ET4 cells. However, despite good expression of the R288H mutant bombesin receptor in Balb 3T3 cells (Ref. 30 and results not shown), the mutated receptor was no longer able to respond to bombesin (Fig. 4B). SpD (3 μM) induced a 2.5-fold stimulation of ERK activity in 5ET4 cells. Crucially, SpD was also no longer able to stimulate an increase in ERK<sub>12</sub> phosphorylation or ERK<sub>13</sub> activity in cells expressing the R288H mutant, although these cells still able to respond to lysophosphatidic acid (results not shown). This confirms that SpD acts at the agonist-binding domain of the GRP receptor to stimulate ERK activation but does not mobilize Ca<sup>2+</sup>.

**GRP Receptor Desensitization**—Previous experiments have shown that GRP receptors undergo desensitization upon exposure to bombesin (35) and that differences in receptor desensitization and down-regulation can result in altered agonist responses. Given the differences in time course for bombesin- and SpD-induced ERK activation we determined whether this could be explained by differential GRP receptor desensitization. [125I]-GRP receptor binding was measured in BOR-15 cells. BOR-15 cells bound [125I]-GRP with an affinity (K<sub>d</sub>) of 0.48 ± 0.11 nM and a B<sub>max</sub> of 0.72 ± 0.08 × 10<sup>6</sup> binding sites/cell (Fig. 5A, inset). SpD inhibited [125I]-GRP binding with an affinity (K<sub>i</sub>) of 3.5 ± 0.11 μM (Fig. 5A), which is in good agreement with its ability to activate ERK (Fig. 2) and JNK (Fig. 6) and confirms that SpD acts via the GRP receptor. [125I]-GRP binding was measured after exposure to maximal inhibitory concentrations of bombesin (30 nM) or SpD (30 μM, Fig. 5B). As expected, pre-exposure to bombesin produced a decrease in receptor binding with a 54 ± 10% reduction after 2 h. In contrast, SpD produced only a 15 ± 7% reduction, which was nonsignificant over this time scale. SpD therefore does not induce receptor desensitization to the same extent as bombesin. This will have important implications for ERK and JNK activation.

**SpD-induced JNK Stimulation Is Blocked by Dominant Negative G<sub>i12</sub>-** We and Larpe et al. (24) have shown previously that substance P analogues activate JNK (21, 25). Evidence suggests that JNK activation may be mediated by members of the G<sub>i12</sub> family of G-proteins such as G<sub>i12</sub> and G<sub>i13</sub> (36, 37). Hence it was hypothesized that SpD acted as an agonist for the G<sub>i12</sub> family of G-proteins. However, Sinnett-Smith et al. (28) recently showed that substance P analogues block bombesin-stimulated assembly of focal adhesion and actin stress fiber formation in Swiss 3T3 cells. G<sub>i12</sub> and G<sub>i13</sub> activation can induce these Rho-mediated events, suggesting that SpD acts as an antagonist for members of the G<sub>i12</sub> family of G-proteins (28).

We therefore went on to examine the effect of directly blocking G<sub>i12</sub> and G<sub>i13</sub> on SpD activation of JNK. We used G<sub>i12</sub> and G<sub>i13</sub> dominant negative constructs that have been shown to block G<sub>i12</sub>- and G<sub>i13</sub>-mediated stress fiber formation in fibroblasts and to have no effect on G<sub>q</sub>- or G<sub>q11</sub>-mediated events (14, 38). The constructs contain a G<sub>a</sub>→A substitution in the nucleotide-binding pocket, which blocks activation of COS-7 cells, which express endogenous G<sub>i12</sub> receptors, were transiently transfected with HA-JNK<sub>1</sub>. After 48 h, the cells were washed, stimulated with peptide for 5 min, and lysed. JNK activity was determined from anti-HA precipitates. SpD caused a concentration-dependent stimulation of JNK activity in COS-7 cells (Fig. 6A). This was evident at 0.3 μM, maximal at 30 μM with
ent experiments. BOR-15 cells bound [125I]-GRP with an affinity \( (K_d) \) of 0.48 ± 0.11 nM and a \( B_{\text{max}} \) of 0.72 ± 0.08 × 10^5 binding sites/cell. Desensitization of the GRP receptor after bombesin or SpD pretreatment. Confluent cultures of BOR-15 cells were washed once in PBS and incubated in DMEM containing 1 mg/ml bovine serum albumin and was routinely 35–45% of the total.

Cells were washed three times in cold PBS, solubilized in 0.1N NaOH/2% NaCO3/1% SDS, and counted for [125I]-GRP binding in BOR-15 cells. BOR-15 cells were incubated with [125I]-GRP (0.01 μCi and 1 nM unlabeled GRP) for 30 min at 37 °C. Bound radioactivity was determined as described for [125I]-GRP binding in BOR-15 cells. BOR-15 cells were incubated with either 30 nM bombesin (30 nM-100 nM) or bombesin (3 nM). HA-JNK1 was precipitated with an anti-HA antibody, and kinase activity was measured by phosphorylation of phosphorylated ERK1/2, showed that as expected, control), the dominant negative G\(_{12}\) (G225A) and HA-tagged JNK. COS-7 cells were stimulated with SpD (30 μM) or bombesin (3 nM). HA-JNK was precipitated with an anti-HER antibody, and kinase activity was measured by phosphorylation of glutathione S-transferase-c-Jun (79) and phosphorimaging. The results are expressed as the -fold increase over unstimulated cells and represent the mean ± S.E. of at least three independent experiments performed in duplicate. *, \( p < 0.05 \), analysis of variance. C, aliquots of cell lysate transfected with the dominant negative G\(_{12}\) or lacZ and stimulated with bombesin or SpD were resolved by SDS-PAGE and transferred onto nitrocellulose. The blots were probed with a monoclonal antibody raised to the dually phosphorylated form of JNK1 or JNK2.

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**FIG. 5.** [125I]-GRP receptor binding to BOR-15 cells. A, confluent cultures of BOR-15 cells (30-mm dishes) were incubated with SpD (1 nM-100 μM) and [125I]-GRP (0.01 μCi and 1 nM unlabeled GRP) in DMEM containing 1 mg/ml bovine serum albumin for 30 min at 37 °C. Cells were washed three times in cold PBS, solubilized in 0.1 N NaOH/2% NaCO3/1% SDS, and counted for [125I]-GRP (0.01 μCi and unlabeled GRP (0.01–30 nM) for 30 min at 37 °C. Bound radioactivity was determined as described for A. Results are presented as a Scatchard plot (28) representative of four independent experiments. BOR-15 cells bound [125I]-GRP with an affinity \( (K_d) \) of 0.48 ± 0.11 nM and a \( B_{\text{max}} \) of 0.72 ± 0.08 × 10^5 binding sites/cell. B, desensitization of the GRP receptor after bombesin or SpD pretreatment. Confluent cultures of BOR-15 cells were washed once in PBS and incubated in DMEM containing 1 mg/ml bovine serum albumin and either 30 nM bombesin (●) or 30 μM SpD (□) for various times as indicated. Cells were then washed three times in PBS and incubated with [125I]-GRP (0.01 μCi and 1 nM unlabeled GRP) as described for A. Results are expressed as specific binding (disintegrations/min) and represent the mean ± S.E. of three experiments performed in triplicate.

**FIG. 6.** SpD-induced JNK activation is inhibited by a dominant negative G\(_{12}\). A, COS-7 cells were grown to 80% confluency in 100-mm dishes and serum-starved (0.1% FCS) for 24 h prior to experimentation. Cells were stimulated with SpD for 15 min at 37 °C. Lysates were prepared, and JNKs was assayed from immunoprecipitated JNKs, phosphorylation of 1 μg of glutathione S-transferase-c-Jun (79). Phosphorylated proteins were resolved by SDS-PAGE, and incorporated radioactivity was measured by phosphorimaging. The results represent the mean ± S.E. of three independent experiments. A representative autoradiograph is shown. B, COS-7 cells were grown to 70% confluency and transfected with the pCIS vector containing lacZ (control), the dominant negative G\(_{12}\) (G225A), or the dominant negative G\(_{12}\) (G225A) and HA-tagged JNK. COS-7 cells were stimulated with SpD (30 μM) or bombesin (3 nM). HA-JNK was precipitated with an anti-HER antibody, and kinase activity was measured by phosphorylation of glutathione S-transferase-c-Jun (79) and phosphorimaging. The results are expressed as the -fold increase over unstimulated cells and represent the mean ± S.E. of at least four independent experiments performed in duplicate. *, \( p < 0.05 \), analysis of variance. C, aliquots of cell lysate transfected with the dominant negative G\(_{12}\) or lacZ and stimulated with bombesin or SpD were resolved by SDS-PAGE and transferred onto nitrocellulose. The blots were probed with a monoclonal antibody raised to the dually phosphorylated form of JNK, or ERK1/2. The results are representative of at least three independent experiments.

EC\(_{50}\) = 1.2 μM. SpD- and bombesin-induced JNK activation was blocked completely in COS-7 cells co-expressing the dominant negative \( \alpha \) subunit of G\(_{12}\) (Fig. 6, B and C) but not G\(_{12}\) (Fig. 6B), and thus SpD activates JNK via a stimulation of G\(_{12}\). Measurement of ERK activity, assessed by Western blot analysis of phosphorylated ERK1/2, showed that as expected, concentration response curves to bombesin and SpD-induced ERK activation was completely unaffected by the expression of the dominant negative G\(_{12}\) (Fig. 6C). This suggests that G\(_{12}\) specifically inhibits JNK activation, which is not caused by a general inhibition of receptor function by \( \alpha \) subunits.

**SpD-induced ERK Activation Is Inhibited by Pertussis Toxin**—We examined the G-protein dependence of SpD-induced ERK activation. Fig. 7 shows that ERK stimulation by SpD in BOR-15 cells is inhibited by 24-h pretreatment with pertussis toxin (100 ng/ml). Pertussis toxin, however, had no effect on bombesin-stimulated ERK in accordance with previously reported data (17, 39). These results suggest that unlike bombesin, SpD-induced ERK activation occurs via an activation of G-proteins of the G\(_{12}\) subtype. SpD therefore has the unique ability to inhibit responses mediated by G\(_{12}\) (Ca\(_{\text{2+}}\) mobilization) within the same concentration range that activates G\(_{12}\)-mediated responses.

It has been reported that many G-protein-linked receptors such as bradykinin receptors activate ERK via the transactivation of growth factor receptors such as the epidermal growth factor (EGF) receptor (40, 41). Fig. 8 shows that bombesin-induced activation of ERK in BOR-15 cells was blocked completely by prior treatment for 30 min with the EGF receptor kinase inhibitor AG1478, whereas SpD-induced activation was completely insensitive. These results suggest that bombesin stimulates ERK activation via transactivation of the EGF receptor in contrast to SpD. This confirms that bombesin and SpD differentially modulate G-protein signal transduction via the bombesin receptor.
Substance P Analogues as Biased Agonists

SpD Acts as a Biased Agonist in SCLC Cells—Substance P analogues have been developed for use as anticancer agents in SCLC. We therefore examined whether biased agonism has functional relevance in SCLC cells. Bombesin/GRP has been shown to be a principal autocrine growth factor for the SCLC cell line H345, with bombesin receptor blockade by the antagonist [Leu^13(CH2NH)-Leu^14]bombesin or the monoclonal antibody 2A11 inhibiting growth in vitro and in vivo (3–5, 42). In the H345 cells, bombesin induced a marked and rapid increase in [Ca^{2+}], which was blocked by SpD (Fig. 9A). In addition, SpD at concentrations as high as 100 μM failed to mobilize intracellular calcium in SCLC cells (data not shown). Bombesin induced a concentration-dependent increase in [Ca^{2+}], in H345 cells, with EC_{50} = 9.2 ± 4.2 nM (n = 5). SpD shifted the [Ca^{2+}]_i response curve to bombesin to the right (Fig. 9A, insert). The inhibitory effect of SpD on bombesin (10 nM) is seen first at 3 μM, maximal at 100 μM, with IC_{50} = 12.7 ± 2.5 μM (Fig. 9A). The addition of SpD inhibited the growth of H345 SCLC cells in liquid culture. Growth inhibitory effects were seen first at 3 μM, maximal at 50 μM with IC_{50} = 29.5 ± 5.5 (Fig. 9B). This is in keeping with previously published results (7, 17). We further show that within the concentration range in which SpD inhibits bombesin-stimulated increase in [Ca^{2+}], and growth in H345 SCLC cells, SpD stimulates ERK (Fig. 9C) and JNK activation (Fig. 9D) (EC_{50} = 3.9 ± 1.7 μM and 1.2 ± 2.3, respectively) without increasing [Ca^{2+}]. Thus in the physiologically relevant in vitro cell system, SpD exhibits both antagonist and agonist activity at the same concentrations, consistent with biased agonism.
DISCUSSION

The novel findings in this paper are: 1) SpD binds to the ligand-binding site of the bombesin/GRP receptor and this binding critically depends on amino acid Arg-288. 2) SpD increases ERK and JNK activity via the activation of the bombesin/GRP receptor within the same concentration range that blocks bombesin receptor-mediated increases in [Ca^{2+}], 3) The activation of JNK by SpD and bombesin depends on G_{12} activation. 4) SpD unlike bombesin does not cause GRP receptor desensitization. 5) ERK activation by SpD is pertussis toxin-sensitive unlike ERK activation by bombesin, which is pertussis toxin-insensitive but depends on EGF receptor tyrosine kinase activity.

SpD is a decapeptide analogue of substance P. Compounds of this type (e.g. [Arg^{6}, \text{d}-Trp^{7,9}, N^{methyl}Phe^{8}]substance P (6–11) and [\text{d}-Arg^{1}, \text{d}-Trp^{5,7,9}, Leu^{11}]substance P) have been shown to inhibit the growth of SCLC cells in vitro and in vivo and stimulate SCLC cell apoptosis (22, 23). Traditionally, substance P analogues were thought to inhibit SCLC cell growth by competitively inhibiting the effects of autocrine and paracrine mitogenic neuropeptides (7, 17–20). However, substance P analogues are not simply acting as competitive neuropeptide antagonists. Our data demonstrate that SpD has the unique ability to allow the bombesin receptor to couple to G_{i} and G_{12p} leading to subsequent ERK and JNK activation, respectively, while at the same time blocking receptor activation of G_{i} mediated calcium release. SpD is the first compound shown to be capable of activating and inactivating different arms of the signal transduction pathways activated by a single receptor. Thus SpD is acting as a biased agonist at the GRP receptor and our results provide a formal validation of this novel pharmacological term.

Studies with synthetic bombesin-like peptides have demonstrated that full biological activity requires more than seven but no more than nine N-terminal amino acids (WAVGHLMN); on this basis it is proposed that these N-terminal residues interact with the bombesin receptor ligand-binding pocket defined by residues Gln-121, Arg-288, Ala-308, and Pro-199 (33). The R288H GRP receptor mutant shows a 1000-fold reduction in GRP/bombesin affinity relative to the wild-type mouse GRP receptor (33). SpD-induced ERK activity was inhibited by two specific GRP receptor antagonists and was abrogated in cells expressing the mutant (R288H) GRP receptor. Thus the biased agonist activity of SpD is mediated via binding to the ligand-binding site of the bombesin/GRP receptor. Substitution of \text{d}-Phe for Gln at position 5 in SpD results in a complete loss of activity against the bombesin receptor (result not shown). Hence a large nonpolar hydrophobic residue \text{d}-Phe in SpD and Trp in bombesin at a position of seven amino acids from the N terminus may be critical for optimal binding to the ligand-binding pocket.

We show that despite binding to the agonist-binding domain of the bombesin/GRP receptor, SpD is unable to produce a Ca^{2+} response but can inhibit bombesin-induced Ca^{2+} mobilization, which is mediated via G_{i}-dependent stimulation of phospholipase C-\beta. Bombesin on the other hand stimulates JNK and ERK and increases [Ca^{2+}], However, SpD-induced ERK stimulation is prolonged compared with bombesin. These kinetics are similar to that observed for SpD-induced activation of JNK, which also has a protracted time course (21, 24). This suggests that SpD, upon binding to the ligand-binding site, differentially regulates signal transduction pathways downstream from the bombesin/GRP receptor compared with the natural agonist. The mechanisms for this are unclear, but desensitization studies showed that SpD is much less efficient at desensitizing the receptor compared with bombesin. This has important functional implications. It could suggest that bombesin cannot fully activate all possible receptor–G-protein interactions because it causes rapid desensitization. Previous evidence suggests that GRP receptors cause rapid ligand degradation, which can account for much of the observed desensitization caused by bombesin (35). SpD therefore may stabilize an active conformation not activated by bombesin (e.g. G_{i}) because of a longer-lived association with the receptor.

Evidence suggests that JNK activation may be mediated by members of the G_{12} family of G-proteins such as G_{12} and G_{13}. Hence it was hypothesized that SpD acts as an agonist for the G_{12} family of G-proteins (25, 36, 37). Recently, Sinnett-Smith et al. showed that substance P analogues block bombesin-stimulated assembly of focal adhesions and actin stress fiber formation and only showed activation of these responses at high concentrations, suggesting that they were acting as partial agonists (28). G_{12} and G_{13} activation can induce these Rho-mediated events, suggesting that SpD acts as an antagonist for members of the G_{12} family of G-proteins. However, Jarpe et al. (24) showed that substance P analogues could stimulate the assembly of focal adhesion and an increase in actin stress fibers in Swiss 3T3 cells. We show that in the same samples, SpD activates JNK and ERK at the same concentrations that inhibit bombesin-stimulated Ca^{2+} mobilization (EC_{50} for JNK and ERK activation is 4.2 and 3.2 \mu M, respectively, and IC_{50} for inhibition of bombesin-induced Ca^{2+} mobilization = 3.7 \mu M). These data would not be consistent with partial agonism but suggest that SpD can induce a conformational state that favors coupling and activation of some but not all G-protein subtypes. We examined the effect of directly blocking G_{12} and G_{13} on SpD activation of JNK. Both bombesin and SpD activation of JNK is blocked by dominant negative G_{12} but not by a dominant negative G_{13}. Neither of these dominant negative G-proteins affect ERK activation. Therefore we have shown that JNK stimulation by SpD is mediated via G_{12} activation, and crucially, this occurs within the same concentration range, which inhibits bombesin-induced G_{i} activation.

The mechanisms by which neuropeptides activate ERK are diverse and cell type-dependent (41). In this study we showed that bombesin-stimulated ERK activation was inhibited by the EGF receptor tyrosine kinase inhibitor AG1478 but was pertussis toxin-insensitive. This finding is consistent with previous studies on G-protein-coupled receptor-induced ERK activation in rat-1a cells, which demonstrate that transactivation of growth factor receptors is required for ERK activation by G-protein-coupled receptors (41). Previous results have also shown that bombesin inhibits [^{125}I]-EGF binding to its receptor (43), implicating EGF receptor activation in bombesin-mediated signaling. However, we also showed that SpD-induced ERK activation was not blocked by AG1478 but was blocked by pertussis toxin. ERK activation by G_{i} receptors in many cases has been shown to be caused by the liberation of \beta_{y} subunits from G_{i} that can directly activate phosphatidylinositol 3-kinase, leading to the activation of Ras and the Raf/MEK/ERK cascade (44). However there is evidence in Jurkat T lymphocytes that G_{i} proteins can activate ERK via a Ras and phosphatidylinositol 3-kinase-independent pathway that is mediated by the G_{ai} subunit (45). Whatever the case, our data suggest that SpD has the unique ability to differentially activate signal transduction pathways at the agonist-binding site, allowing bombesin receptors to couple to G_{i} proteins, leading to subsequent ERK activation while at the same time blocking receptor activation of G_{q}–stimulated phospholipase C activation and Ca^{2+} release.
SpD is the first compound shown to be capable of simultaneously activating and inactivating different arms of the signal transduction pathways normally activated by a full agonist. This is not caused by SpD favoring activation of G-proteins with faster rates of GDP/GTP exchange, because dissociation of GDP from G12 is 10–20-fold slower than other α subunits (46). Furthermore, the prolonged kinetics of both JNK and ERK activation and the reduced ability of SpD to desensitize the GRP receptor compared with bombesin support the concept that SpD is bound to the receptor for a long enough period to activate all potential family members of G-proteins. It would seem likely from our observations that SpD is not acting as a partial agonist. Partial agonists bind to the receptor and compete with the agonist for binding but have lower efficacy than a full agonist; however, they should activate all the same signal transduction pathways as the natural ligand with the same time course. Our data therefore suggest that SpD, upon binding to the bombesin receptor, causes a conformational change that differentially activates G-proteins.

For agonist-receptor trafficking (26, 27) it has been suggested that structurally different agonists may occupy the receptor, causing different active receptor conformations that selectively activate G-proteins (47, 48). Therefore, the diversity of agonist responses in different cell types may not be solely caused by cellular differences in receptor/G-protein stoichiometry. The cubic ternary complex model (Fig. 10) allows for the existence of multiple active and inactive receptor states, with agonists stabilizing the formation of multiple states via affinity factors α, β, and γ (47). Although designed to describe the efficacies of different agonists, this model can be extended to explain equally well the action of a biased agonist that promotes the existence of activation states that stimulate certain G-proteins (e.g. G1 and G12 for SpD) but not others or even stabilizes an inactive state (inhibition of Gq). In this model the final response given by an agonist will be governed by its promotion of active states and the relative amounts of G-protein available for stimulus-response coupling. Biased agonism therefore extends the idea of agonist-receptor trafficking to include compounds that activate and inactivate different receptor/G-protein conformations.

There is a structural basis to support the concept that different conformational states result in selective G-protein activation by a receptor. Deletion of part of the seventh transmembrane domain of the calcitonin receptor favors Gα coupling over Gq (49). Mutations in the thyrotropin receptor uncouple the receptor from the G-protein that mediates phospholipase C activation (most likely Gq) while maintaining coupling to Ga (50). Furthermore, mutations in G-protein-coupled receptors result in receptors that are in an active conformational state in the absence of ligand (51).

We provide functional relevance for these observations in one of the most important biological systems in which neuropeptides play a role: SCLC. Our results show that SpD stimulates a sustained activation of JNK and ERK (EC50 = 3.9 ± 1.7 μM and 1.2 ± 2.3, respectively) over the same concentration range that inhibits SCLC cell growth (IC50 = 29.5 ± 5.5) and GRP-stimulated increase in [Ca2+]i (IC50 = 12.7 ± 2.5 μM). At these concentrations SpD also induces apoptosis (21, 23). We propose that biased agonism at the GRP receptor causes discordant signaling (ERK and JNK activation in the absence of Ca2+ mobilization), which results in the full growth inhibitory effect of substance P analogues. Our results suggest that the biased agonist activity of SpD subverts the cancer cells growth factor receptors from stimulating proliferation to initiating programmed cell death in addition to blocking the effects of mitogenic neuropeptides. This is an exciting new concept in cancer chemotherapy.

The results presented here provide a unifying mechanism to explain the previously published observations using substance P analogues. SpD activates the GRP/bombesin receptor in such a way as to cause association with only a subset of possible α subunits; this is the first agonist shown to act in this manner. SpD binds to the ligand-binding site of the GRP receptor and will therefore inhibit ligand-receptor binding (7, 17, 18) and act as a competitive GRP antagonist for certain responses mediated via Gαq (7, 17, 19, 20). However, SpD also has activity that cannot be explained by competitive antagonism alone. In Swiss 3T3 cells where SpD and [α-Arg16,γ-Trp7,9,Leu11] substance P inhibit bombesin-stimulated ERK activity (presumably via blocking Gαq (18, 25)), SpD also augments Raf and ERK activity at higher bombesin concentrations (25), which we propose results from the activation of Gαq. In different cells the degree of stimulation of Gαq-mediated ERK activation is likely to depend on the level and ratio of receptor and G-protein expression (41). The SCLC cell growth inhibitory and proapoptotic activities of substance P analogues are not reversed by supramaximal saturating concentrations of neuropeptides (21). In terms of conventional pharmacology, this is incompatible with competitive

![Biased agonist hypothesis](image-url)
antagonism. However, for transient signaling events such as intracellular calcium mobilization, SpD acts as a competitive antagonist and is reversible with bombesin. Longer term effects lead to the activation of programmed cell death, which is irreversible. Thus, once apoptosis has been initiated by SpD this cannot be reversed by excess agonist.

Our characterization of the mechanism of action of SpD can be used further for future drug development. It also offers the opportunity to modulate neuropeptide receptor signaling to activate some signal transduction pathways while blocking others. Bombesin/GRP receptors are potential therapeutic targets for the treatment of a number of human disease states including obesity, inflammation, and cardiovascular disease as well as cancer. Thus modulation of neuropeptide signaling has important clinical implications. The majority of pharmacological work for the treatment of human disease has focussed on specific receptor blockade with minimal side effects; however, even specific receptor blockade can lead to deleterious side effects. With the establishment of the concept of biased agonism much more specific pharmacological agents could be generated that will selectively affect specific receptor downstream signal transduction pathways developing both receptor-specific and signal transduction-specific pharmacological agents.

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Bombesin and Substance P Analogues Differentially Regulate G-protein Coupling to the Bombesin Receptor: DIRECT EVIDENCE FOR BIASED AGONISM
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