[\text{\text{D-Arg}^1,\text{D-Phe}^5,\text{D-Trp}^7,9,\text{Leu}^{11}}]\text{Substance P Acts as a Biased Agonist toward Neuropeptide and Chemokine Receptors}\text{*}

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Substance P derivatives are potential therapeutic compounds for the treatment of small cell lung cancer and can cause apoptosis in small cell lung cancer cells in culture. These peptides act as broad spectrum neuropeptide antagonists, blocking calcium mobilization induced by gastrin-releasing peptide, bradykinin, cholceystokinin, and other neuropeptides. We show that [\text{D-Arg}^1,\text{D-Phe}^5,\text{D-Trp}^7,9,\text{Leu}^{11}]\text{Substance P has unique agonist activities in addition to this described antagonist function. At doses that block calcium mobilization by neuropeptides, this peptide causes activation of c-Jun N-terminal kinase and cytoskeletal changes in Swiss 3T3 fibroblasts and stimulates migration and calcium flux in human neutrophils. Activation of c-Jun N-terminal kinase is dependent on the expression of the gastrin-releasing peptide receptor in rat 1A fibroblasts, demonstrating that the responses to the peptide are receptor-mediated. We hypothesize that [\text{D-Arg}^1,\text{D-Phe}^5,\text{D-Trp}^7,9,\text{Leu}^{11}]\text{Substance P acts as a biased agonist on neuropeptide and related receptors, activating certain guanine nucleotide-binding proteins through the receptor, but not others.}

Neuropeptides are a structurally diverse group of hormones and neurotransmitters that bind to a related subfamily of G protein-coupled receptors (1) and function in neuron-to-neuron communication, as well as in signaling in the immune system and in tissue restructuring. Neuropeptides and their receptors are the principal driving force behind one of the most clinically aggressive cancers, small cell lung cancer (SCLC).1 SCLC tumors sustain their growth, in part, by maintaining neuropeptide autocrine and paracrine loops (2). These tumor cells in culture can secrete and respond mitogenically to multiple neuropeptides (3). For this reason, broad spectrum antagonists of neuropeptides have been examined for their ability to prevent growth of SCLC cells in vitro and in vivo. One such compound, [\text{D-Arg}^1,\text{D-Phe}^5,\text{D-Trp}^7,9,\text{Leu}^{11}]\text{Substance P (SPD-D) (4) can not only inhibit the action of Substance P (5) but also inhibit binding and action of gastrin-releasing peptide (GRP), arginine vasopressin (6), and endothelin (7). To date, much of the research on the mechanism of action of SPDs has focused on their abilities to block ligand binding and \text{Ca}^{2+}\text{ flux, as well as on their cytostatic or cytotoxic effects on SCLC cells in culture (8).}

The signal transduction pathways that mediate neuropeptide actions are rapidly becoming more clear. It is known that neuropeptides induce calcium mobilization by a pertussis toxin-insensitive mechanism, suggesting a role for \text{G}_{\alpha}(9). Heterologous expression of receptors and G proteins in S9F cells have shown functional coupling between one neuropeptide receptor, NK-1, and \text{G}_{\alpha}, \text{G}_{\beta}, and \text{G}_{\gamma} but not \text{G}_{\delta} (10). In addition to the mobilization of \text{Ca}^{2+} from intracellular stores, neuropeptides can have diverse effects on cells in culture, including the induction of mitogenesis (11), the activation of both the extracellular signal-regulated kinase (ERK) (12) and the c-Jun N-terminal kinase (JNK) (13, 14) members of the mitogen-activated protein kinase family, and formation of actin structures such as filopodia (15) and stress fibers (16, 17). The activation of JNK and the reorganization of the actin cytoskeleton are most likely mediated by members of the \text{G}_{\beta}\gamma family of G proteins, such as \text{G}_{\alpha}12 and \text{G}_{\alpha}13 (18, 19). The regulation of ERKs by neuropeptides is more complicated, suggesting a role for PKC (20), G protein \beta\gamma subunits (12), and a pertussis toxin-sensitive mechanism possibly involving a \text{G}_{\delta} family member (21).

The effects of SPD-D and similar compounds on these signaling events downstream of neuropeptide receptors suggest a mechanism that is more complicated than that of a classical antagonist. The ERK response and the \text{Ca}^{2+} response through neuropeptide receptors are affected differently by SPD-D (22, 23). In Swiss 3T3 fibroblasts, SPD-D inhibits \text{Ca}^{2+} mobilization induced by bombesin (which acts on the human GRP receptor) with a maximal effect at 10 \text{μM} and an estimated IC_{50} of 2 \text{μM}. In contrast, SPD-D inhibits ERK-2 activation with a maximal effect at 50 \text{μM} and an estimated IC_{50} of 9 \text{μM} (22). In the presence of 3 \text{nM} bombesin, 10 \text{μM} SPD-D caused a nearly complete inhibition of the \text{Ca}^{2+} response and an approximately 50\% inhibition of the ERK-2 response (22). In a similar manner, 50 \text{μM} SPD-D caused a 100\% inhibition of the inositol 1,4,5-trisphosphate generation induced by 50 \text{nM} bombesin, but only a 20\% inhibition of the total ERK response (23).

In an effort to uncover the mechanism behind these differences, we examined the effect of SPD-D on other signal transduction pathways downstream of neuropeptide receptors, such as the activation of JNK and rearrangement of the actin cy-
toskeleton. To our surprise, rather than acting to inhibit these responses caused by bombesin, SPD-D stimulated both JNK and cytoskeletal changes. These responses are shown to occur through the action of SPD-D on neuropeptide receptors, because rat 1A cells, which do not respond to SPD-D with a JNK response, gain the ability to respond when transfected with the GRP receptor. SPD-D can also act as an agonist toward chemotactrant receptors in human neutrophils. We show that SPD-D binds to both of the cloned interleukin-8 (IL-8) receptors, and the peptide can stimulate an increase in neutrophil migration and Ca\(^{2+}\) mobilization.

These results indicate that the mechanism of action of SPD-D is more complex than has previously been appreciated. Rather than acting as a classical antagonist, SPD-D is capable of initiating signals in multiple cell types in a receptor-dependent manner. Unlike a classical agonist, however, SPD-D does not induce receptor signaling to all available signal transduction pathways. SPD-D selectively activates JNK and actin reorganization in fibroblasts in the absence of Ca\(^{2+}\) mobilization. This is the first description of such a biased agonist. We expect that this novel mechanism of action will prove an important component of the cytototoxic activity such compounds have on SCLC. An understanding of these mechanisms is crucial to the development of this type of therapy for future clinical use.

### EXPERIMENTAL PROCEDURES

**Materials**—bpArg\(^1\)-D-Phe\(^2\)-D-Trp\(^7\)-Leu\(^11\))substance P, purchased from Bachem BioScience, was stored in powder form at \(-20^\circ\)C and dissolved to 20 mM in degassed, sterile water and stored under argon for no more than 4 weeks. Rhodamine-conjugated phalloidin was purchased from Molecular Probes. Rabbit anti-β-galactosidase antibody was purchased from Cappel. Recombinant human IL-8 was purchased from Boehringer Mannheim Biochemicals. Recombinant human IL-8 was purchased from R&D Systems, recombinant C5α was from Sigma, and [Tyr\(^4\)]bombesin was from Peninsula Laboratories. 125I-Labeled C5α, IL-8, and bombesin were purchased from NEN Life Science Products. Materials—[D-Arg\(^1\),D-Phe\(^5\),D-Trp\(^7\),9,Leu\(^11\)]substance P, purchased from Bachem BioScience, was stored in powder form at \(-20^\circ\)C and dissolved to 20 mM in degassed, sterile water and stored under argon for no more than 4 weeks. Rhodamine-conjugated phalloidin was purchased from Molecular Probes. Rabbit anti-β-galactosidase antibody was purchased from Cappel. Recombinant human IL-8 was purchased from R&D Systems, recombinant C5α was from Sigma, and [Tyr\(^4\)]bombesin was from Peninsula Laboratories. 125I-Labeled C5α, IL-8, and bombesin were purchased from NEN Life Science Products.

**Cell Lines and Culture**—Swiss 3T3 fibroblasts were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum (DME-10% FCS) and the actin cytoskeleton was stained with rhodamine-phalloidin, 10 μg/ml phallolidin, 10 μg/ml rhodamine-phalloidin, and 10 μg/ml rhodamine-phalloidin. The cells were washed and intracellular calcium was determined by imaging on a TopCount scintillation counter (Packard).

### RESULTS

**SPD-D Induces JNK in Fibroblasts**—Bombesin, a peptide agonist of the GRP receptor, was found to stimulate JNK activation in Swiss 3T3 fibroblasts (Fig. 1A) with a dose response that is similar to the stimulation of ERK activity (23) in these cells (data not shown). 50 μM SPD-D was used in previous studies to inhibit inositol 1,4,5-trisphosphate generation and ERK activation by bombesin (23). Rather than inhibiting bombesin-stimulated JNK activation, 50 μM SPD-D caused a similar activation of JNK (Fig. 1A), which reached a maximum at 5 min after stimulation (Fig. 1B). This activity was sustained for 30 min and returned to baseline by 60 min (data not shown). The dose response curve for JNK activation is shown in Fig. 1C. The maximum activation was reached at 40 μM.

The activation of JNK by SPD-D is surprising because thus far this peptide has been described as a competitive antagonist that acts by binding to the receptor and blocking binding of neuropeptide (4). The activation of JNK suggests that the peptide has agonist properties, not unlike similar compounds based on the structure of substance P (27, 28). However, this agonist activity is unique in that SPD-D does not mobilize Ca\(^{2+}\) in Swiss 3T3 cells (22). To test whether this unique agonist property was the result of SPD-D binding to neuropeptide receptors, we used a transient transfection assay in another cell line, rat 1A fibroblasts.

Untransfected rat 1A fibroblasts failed to respond to a dose of SPD-D as high as 50 μM. When these cells were transiently transfected with the human GRP receptor, the basal JNK activity increased (Fig. 2A). This increase in the basal JNK activity is most likely due to spontaneous activity in the expressed receptor, similar to that seen with the bradykinin receptor (29) and the calcitonin receptor (30), which activate adenylate cyclase in the absence of agonist when overexpressed. Cells transiently expressing the GRP receptor gained the ability to respond to SPD-D (Fig. 2A). SPD-D can therefore stimulate JNK activity through the GRP receptor.

Rat 1A cells transfected with the empty vector did not respond to bombesin with an increase in intracellular calcium (Fig. 2B), but when these cells were transfected with the GRP receptor, 50 nM bombesin caused a rapid Ca\(^{2+}\) flux. SPD-D did not stimulate Ca\(^{2+}\) mobilization in rat 1A fibroblasts with or without the GRP receptor (Fig. 2B). This is in agreement with the inability of SPD-D to cause calcium flux in Swiss 3T3 or
SPD-D activates JNK in Swiss 3T3 fibroblasts. A, Swiss 3T3 fibroblasts were starved for 1 day in 0.1% calf serum and treated as indicated with 50 nM bombesin or 50 μM SPD-D for 10 min. Jun kinase activity was determined by precipitation with and phosphorylation of GST-c-Jun. B, time course of SPD-D-induced JNK activation. Swiss 3T3 cells were treated as above with 50 μM SPD-D for the indicated times. The amount of phosphorylation incorporated was quantitated by PhosphorImager analysis, and values are expressed as fold increase over control. An autoradiograph of one representative experiment is shown in the bottom panel. C, cells were treated with the indicated concentrations of SPD-D for 10 min, and Jun kinase activity was determined as above. An autoradiograph of one representative experiment is shown below. Error bars represent 1 S.D. of three (B) or two (C) samples.

SPD-D induces JNK activation but not Ca²⁺ flux in rat 1A cells expressing the gastrin-releasing peptide receptor. A, rat 1A cells were transfected with empty vector (pCMV5) or vector containing the cDNA for the human GRPR. Cells were starved and treated with or without SPD-D, and Jun kinase was determined as described in the legend for Fig. 1. Error bars represent 1 S.D. of four samples. An autoradiograph of one representative experiment is shown in the bottom panel. B, rat 1A cells transfected with pCMV5 (top panel) or pCMV5-human GRPR (bottom panel) were treated with 50 nM bombesin (circles) or 50 μM SPD-D (squares). Calcium flux was measured by laser confocal microscopy using Fluo-3 as a calcium-sensitive dye. Curves are representative of two to four independent experiments. C, rat 1A fibroblasts transiently transfected with the GRP receptor were stimulated with 30 nM bombesin (top), 300 nM bombesin (middle) or 10% fetal calf serum (bottom) in the absence (filled squares) or presence (open squares) of 50 μM SPD-D. Calcium flux was measured as in B.

SCLC cells (4, 22). In these systems, SPD-D has been described as a competitive antagonist of bombesin and other neuropeptides. To confirm this competitive antagonism, we tested the ability of SPD-D to inhibit bombesin-stimulated calcium flux in receptor-expressing rat 1A cells (Fig. 2C). Whereas 50 μM SPD-D was able to inhibit calcium mobilization induced by 30 nM bombesin, 300 nM bombesin was able to overcome this inhibition. Neuropeptide-induced Ca²⁺ mobilization in fibroblasts is mediated by members of the Gq family of Gα subunits (9), whereas it is likely that JNK activation is mediated by Gi12 family members (31). SPD-D therefore acts as a biased agonist, activating Gi12 and subsequent signaling components, without activating Gq and its downstream effectors.

An alternative explanation for the lack of Ca²⁺ mobilization by SPD-D is that SPD-D activates a receptor that initiates a signal transduction pathway that inhibits Ca²⁺ mobilization. To test this, we treated rat 1A cells with 10% fetal calf serum. Serum induced a rapid, sustained Ca²⁺ flux, which was not inhibited by SPD-D in untransfected cells (data not shown) or in cells expressing the GRP receptor (Fig. 2C). This and the fact that 300 nM bombesin can stimulate calcium mobilization in the presence of 50 μM SPD-D show that SPD-D inhibition of calcium mobilization is through competitive antagonism.

SPD-D Induces Actin Cytoskeletal Reorganization in Fibroblasts—In addition to activating JNK, bombesin induces the formation of actin stress fibers in Swiss 3T3 cells (17). Other neuropeptides also cause reorganization of the actin cytoskeleton. For example, bradykinin causes the formation of peripheral microspikes and filopodia in Swiss 3T3 cells (15). Like JNK activation, this cytoskeletal reorganization is mediated by the Gi12 family of G proteins (19). To further test the idea that SPD-D can activate Gi12 in the absence of Gq activation, we visualized the actin cytoskeleton in cells treated with SPD-D. When 10 μM SPD-D was added to quiescent, starved Swiss 3T3 fibroblasts for 10 min, structures formed at the periphery of the cell outside the cortical actin ring (Fig. 3C, arrow). These structures resembled peripheral actin microspikes as they were described in Swiss 3T3 cells treated with bradykinin (15). Some cells showed the beginnings of stress fibers forming at the periphery (Fig. 3D, arrow).

At 25 μM SPD-D, structures resembling filopodia formed (Fig. 3E, arrow). Significant lamellar actin structures were found in most cells at this dose (Fig. 3F). These structures are two-dimensional, as opposed to membrane ruffles, in which the edge of the cell lifts off of the coverslip. At 50 μM SPD-D, adhesion plaques (Fig. 3G), as well as lamellar actin structures (Fig. 3H), formed. At concentrations up to 100 μM, the cells retained their ability to exclude trypan blue dye for up to 20 min (data not shown). This effect of SPD-D on the cytoskeleton
again demonstrates that SPD-D acts as a biased agonist, stimulating G₁₂ signal transduction pathways.

Previous work by this laboratory has mapped out the sequence of events by which G₁₂ can induce cytoskeletal reorganization (19). This response is mediated by the low molecular weight GDP-binding protein Rho. Therefore, if Rho is inactivated by injection of C3 toxin into the cell or inhibited by the expression of a competitive inhibitory mutant of Rho, N19Rho-A, G₁₂-induced stress fiber formation does not occur. To further show that SPD-D-induced responses, such as actin stress fiber formation, are mediated by G₁₂, a plasmid encoding N19Rho-A was microinjected into Swiss 3T3 fibroblasts along with a second construct encoding β-galactosidase. Injected cells, identified by staining for β-galactosidase, could no longer form stress fibers in response to SPD-D (Fig. 3), whereas the neighboring cells that were not microinjected formed stress fibers. This laboratory has previously shown that microinjection of β-galactosidase alone does not interfere with actin stress fiber formation (19). The effect of N19Rho-A on cytoskeletal reorganization provides further evidence that SPD-D acts on the cells through G₁₂.

**SPD-D Binds to IL-8 and GRP Receptors**—SPD-D has been shown to bind to many neuropeptide receptors, acting as a broad spectrum antagonist (3). These receptors are related to one another by sequence homology in a large subfamily. Also members of this family are non-neuropeptide receptors such as those for α-thrombin, C5a, formylmethionylleucylphenylalane, and IL-8 (32). In an effort to determine how broad the receptor specificity of SPD-D was, we tested binding of SPD-D to two of these receptors, as well as to the human GRP receptor. Receptor-transfected cells were used to make membrane fractions for direct binding assays. As shown in Fig. 5, SPD-D can compete for binding of IL-8 to the human CXCR1 with an IC₅₀ of 12.8 μM. In agreement with the work of other groups (4), SPD-D inhibits binding of bombesin to the human GRP receptor.

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FIG. 3. SPD-D causes the reorganization of the actin cytoskeleton in Swiss 3T3 fibroblasts. Swiss 3T3 cells were grown on glass coverslips and starved for 1 day in 0.1% calf serum, followed by 1 day of starvation in 0.1% bovine serum albumin. Cells were treated for 10 min with control medium (A and B), 10 μM SPD-D (C and D), 25 μM SPD-D (E and F), or 50 μM SPD-D (G and H). Cells were then fixed in paraformaldehyde. The actin cytoskeleton was stained with rhodamine-phalloidin and visualized on a digital confocal fluorescence microscope using a × 62 objective. Representative fields are shown for each concentration of SPD-D. Arrows indicate features discussed in the text.

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able to respond to a much lower dose, as low as 1.5 μM (Fig. 6).

This suggests either that the receptor that mediates this response in neutrophils has a higher affinity for SPD-D than either neuropeptide receptors or the IL-8 receptors or that the chemotaxis assay is much more sensitive than the competition binding analysis or the JNK activation. The identity of the receptor that mediates neutrophil chemotaxis in response to SPD-D is not known. Both the IL-8 receptors and the substance P receptor NK-1 are candidates.

SPD-D Induces Ca\textsuperscript{2+} Mobilization in Human Neutrophils—IL-8 and substance P also cause a mobilization of Ca\textsuperscript{2+} in neutrophils. The mechanism for this Ca\textsuperscript{2+} mobilization is different from that elicited by neuropeptides in fibroblasts, in that it is sensitive to pertussis toxin treatment (35, 36). The pathway that leads to IL-8 and substance P-induced Ca\textsuperscript{2+} flux is the activation of a G\textsubscript{i} family member, which releases βγ subunits that activate PLCβ2 and PLC β3 (35).

50 μM SPD-D was found to be capable of causing a release of Ca\textsuperscript{2+} from intracellular stores in human neutrophils (Fig. 7B). This Ca\textsuperscript{2+} flux is comparable to that caused by IL-8 (24). In contrast to the induction of chemokinesis, a low dose of SPD-D, 1.5 μM, was incapable of activating Ca\textsuperscript{2+} flux (Fig. 7A). This suggests either that the receptors involved in the induction of chemokinesis have a higher affinity for SPD-D than do the receptors involved in the Ca\textsuperscript{2+} response, or that the chemotaxis assay is much more sensitive than the Ca\textsuperscript{2+} mobilization assay. Either response could be mediated by one of the known receptors, either IL-8 receptors, NK-1, or another SPD-D receptor that is yet to be described.

As mentioned above, the Ca\textsuperscript{2+} response to IL-8 and substance P is sensitive to pertussis toxin (36), suggesting the involvement of a G\textsubscript{i} family member. The SPD-D-induced Ca\textsuperscript{2+} mobilization in neutrophils is also sensitive to pertussis toxin pretreatment (data not shown). These data therefore suggest that SPD-D can cause receptor association with G\textsubscript{i2}.

**DISCUSSION**

Compounds based on the mechanism of action of SPD-D may one day be used to treat small cell lung cancer. SPD-D causes apoptosis in SCLC cells in culture through an unknown mechanism. Part of this mechanism undoubtedly involves interruption of autocrine loops and prevention of calcium signaling. However, as we have shown in this report, SPD-D can act as an agonist against receptors that are expressed in SCLC tumors. This agonist activity introduces a signal, while the antagonist activity of the peptide serves to block the Ca\textsuperscript{2+} response. This asymmetric signal transduction may cause a perturbation in cellular processes that leads to apoptosis.

The agonist activity of SPD-D is unique for two reasons. First, SPD-D binds to multiple receptors that recognize diverse ligands. By comparing the structures of other substance P derivatives and examining the biological activity of these com-
response is also seen in other cell systems. SPD-D has never been shown to induce inositol 1,4,5-trisphosphate generation (23). How- ever, in human neutrophils, which express multiple recep-
tors that bind SPD-D, we show that SPD-D induces a JNK activation that is dependent on the expression of the GRP receptor, but it does not induce a Ca\textsuperscript{2+} flux in fibroblasts. Work done in other laboratories supports these data. 10 \mu M SPD-D by itself does not cause Ca\textsuperscript{2+} flux in fibroblasts (22) and 50 \mu M SPD-D does not induce inositol 1,4,5-trisphosphate generation (23). However, SPD-D reliably induces JNK activation at a concentration as low as 10 \mu M. In contrast, a maximal dose of bombesin (4) and has no ability to stimulate JNK or cytoskeletal rearrangement in Swiss 3T3 cells (data not shown). Spantide I contains a glutamine at position 5, whereas SPD-D contains a D-phenylalanine at this position. This suggests that residue 5 in SPD-D is critical in determining biological activity.

The second way in which SPD-D is unique as a ligand is that not all of the possible signals downstream of the receptors are initiated. This is difficult to demonstrate in Swiss 3T3 fibroblasts or in human neutrophils, which express multiple receptors that bind SPD-D. Because SPD-D can bind multiple known and uncharacterized receptors, it is only in a system in which one relevant receptor is expressed that biased agonism can be clearly shown. In rat 1A fibroblasts, we show that SPD-D induces a JNK activation that is dependent on the expression of the GRP receptor, but it does not induce a Ca\textsuperscript{2+} release when this receptor is present. Bombesin, on the other hand, stimulates both JNK and Ca\textsuperscript{2+} flux in fibroblasts. Work done in other laboratories supports these data. 10 \mu M SPD-D by itself does not cause Ca\textsuperscript{2+} flux in fibroblasts (22) and 50 \mu M SPD-D does not induce inositol 1,4,5-trisphosphate generation (23). However, SPD-D reliably induces JNK activation at a concentration as low as 10 \mu M. In contrast, a maximal dose of bombesin stimulates a rapid Ca\textsuperscript{2+} flux and JNK activation. This unique response is also seen in other cell systems. SPD-D has never been shown to flux Ca\textsuperscript{2+} in SCLC cells, but it is capable of blocking bombesin-induced Ca\textsuperscript{2+} flux (2), and it can activate a JNK response in these cells.

SPD-D, therefore, does not fit the definition of either an agonist or an antagonist, so a new model and new terminology are needed to describe the action of this compound. A model of the way in which ligand and receptor interact has been proposed that differentiates between a competitive antagonist and an inverse agonist (37). The model assumes that a receptor can cycle between an inactive (R) and an active (R\textsuperscript{a}) configuration spontaneously in the absence of ligand. This spontaneous activation is the likely mechanism behind increased basal activity of transfected receptors (Fig. 2 and Refs. 29 and 30). A true agonist acts to stabilize the active forms of the receptor, thus causing the receptor to remain in those states for longer and increasing the chance that the receptor will activate a G protein. This model is termed conformational selection (38).

A slight modification of this model can explain the activation of multiple G proteins (Fig. 8) In this model, when a receptor is capable of activating two different G proteins, three states of receptor activation exist: R, the unactivated receptor, and R\textsuperscript{a} and R\textsuperscript{b}, the activated states. R is incapable of activating any G protein; R\textsuperscript{a} can activate Go\textsubscript{q} for example, and R\textsuperscript{b} can activate Go\textsubscript{12}. The receptor cycles between these three states, energetically favoring the inactive state. The existence of more than one active state of the receptor has been postulated based on a phenomenon called agonist trafficking (39). In systems in which a receptor is capable of coupling with multiple G proteins, signals that are downstream of one G protein are often more sensitive to low concentrations of agonist than are signals downstream of another G protein. For example, 0.1 nM calcitonin causes a significant activation of Go\textsubscript{q} through the C1a receptor, whereas it takes up to 10 nM calcitonin to activate Go\textsubscript{q} through this same receptor (30). There are two possible explanations for this difference. Either the Go\textsubscript{q} signal is more easily detected at low levels of receptor activation and thus at lower agonist concentration, or calcitonin can stabilize the form of the receptor that interacts with Go\textsubscript{q} with a greater affinity than it stabilizes the form that activates Go\textsubscript{q}. If the second case is true, there should be agonists that preferentially activate Go\textsubscript{q}. To date, no examples of such a reversal of agonist efficacy have been described for a native receptor.

A competitive antagonist binds to the receptor in any state and competes for binding with the natural ligand. An antagonist may or may not stabilize different states of the receptor. The term “partial agonist” refers to a compound that binds to a receptor and competes with an agonist for binding but itself stabilizes the active states of the receptor to a lesser extent than does a full agonist.

An inverse agonist (29) favors the inactive state and therefore makes it less likely that the receptor will activate a G protein. Inverse agonists decrease the spontaneous activity of the receptor caused by cycling between active and inactive forms, therefore lowering the basal response seen without ligand.

We propose that SPD-D acts in a different manner. SPD-D strongly favors association with R\textsuperscript{b} over R\textsuperscript{a}, resulting in a receptor configuration that activates G\textsubscript{12} but not G\textsubscript{q}. We call such an agonist a biased agonist. This model can be extended to include as many different activation states as there are potential G proteins coupling to the receptor. Our data suggest that SPD-D can cause receptors to couple to Go\textsubscript{q} but not to G\textsubscript{q}. Therefore, SPD-D prefers the states of the receptor that can activate Go\textsubscript{q} and G\textsubscript{12}. Binding to the receptor prevents the bind-

2 M. B. Jarpe and F. M. Mitchell, unpublished observations.
Biased Agonist Properties of a Substance P Derivative

Suggests that a longer association between ligand and receptor compound described to activate receptor-G protein interaction their respective receptors. This broad spectrum of interactions esin, vasopressin, endothelin, IL-8, and other ligands from select for one downstream effector over another.

There is a structural basis for the activation of one set of G proteins by a receptor in the absence of the activation of other G proteins. Mutations in the thyrotropin receptor uncouple the receptor from the G protein that mediates phospholipase C activation (most likely G_q) while maintaining coupling to G_12 (40). Also, a deletion of part of the seventh transmembrane domain of the calcitonin receptor shows a similar change in G protein coupling, favoring G_q over G_12 (30) There is also evidence that certain mutations in G protein-coupled receptors can lead to receptors that can more favorably achieve an active state in the absence of ligand (41).

This hypothesis can be tested by heterologous expression of G proteins and receptors (10). Whereas a classical agonist, such as bombesin, would stimulate the association of the GRP receptor with both Go_q and G12q and a classical antagonist would stimulate association with neither G protein, SPD-D, acting as a biased agonist, would stimulate the association of the receptor with G12q but not Go_q. If so, SPD-D would be the first compound described to activate receptor-G protein interaction selectively. The IL-8 receptors are coupled to members of the G_q class and to members of the G_12 class (35). It would therefore be expected that SPD-D might cause the IL-8 receptor to couple to G_q.

In addition to biased agonism, there are two other possible mechanisms for the selective activation of signaling pathways by SPD-D. First, one of the receptors to which SPD-D binds may generate a signal that can suppress the C_q2^+ response. Second, a difference in the kinetics of G protein activation may select for one downstream effector over another.

SPD-D has the unique property of associating with multiple G protein-coupled receptors. This peptide can displace bombesin, vasopressin, endothelin, IL-8, and other ligands from their respective receptors. This broad spectrum of interactions leaves open the possibility that the lack of C_q2^+ signaling through neuropeptide receptors in fibroblasts and SCLC cells is due to a negative signal from an as yet uncharacterized receptor. However, a high dose of bombesin, or an unrelated agonist, such as fetal calf serum, can still stimulate C_q2^+ mobilization in the presence of SPD-D. This shows that a second signal does not simply shut down calcium mobilization in SPD-D treated cells.

The kinetics of binding between ligand and receptor can vary greatly. It is possible that SPD-D has a much faster on/off rate than bombesin association with the GRP receptor. If this were true, the complex of receptor and SPD-D would be short lived. Only G proteins that are quickly activated would be stimulated by such a short lived complex. SPD-D would therefore favor the activation of one G protein over another without necessarily favoring one active conformation over another. However, the difference in GDP/GTP exchange rate between G12 family members and other G proteins would seem to suggest a bias in the other direction. The rate of dissociation of GDP from G12 is 10–20-fold slower than that for other α subunits (42). This suggests that a longer association between ligand and receptor would be required to activate G12 family members than G_q family members. If SPD-D bound to receptor only briefly, it would therefore be more likely to activate G_q than G_12.

If SPD-D does activate receptors in such a way as to cause association with only a subset of possible α subunits, it would be the first agonist described to act in this manner. Such a biased agonist would become a valuable tool in the dissection of signal transduction pathways downstream of neuropeptide receptors. The concept can also be extended to the treatment of human diseases. Much work has been focused on the search for specific antagonists to receptors in an effort to achieve a clinical outcome without producing side effects. If it becomes possible to selectively affect signal transduction pathways downstream of those receptors, much more specific pharmacological agents could be generated. To pursue this possibility, peptides that are both receptor-specific and pathway-specific would have to be developed. SPD-D binds to several different receptors, which have activity in different physiological processes and may prove to be a biased agonist on some of these receptors and an agonist or antagonist on others.

The original goal of this research was to resolve the mechanism for the differential inhibition of neuropeptide-induced Ca_2^+ responses and ERK activation. In Swiss 3T3 fibroblasts, G proteins of all four major classes are present. Neuropeptide receptors have been shown to directly couple to three of these classes, G_q, G_12, and G_12 (10). The activation of C_q2^+ mobilization proceeds mainly through G_q (9) and JNK activation by neuropeptides may proceed through G_12 (18).

The activation of ERKs by neuropeptides proceeds by a more complex mechanism, with potential roles for PKC (20), βγ subunits (12), and a pertussis toxin-sensitive signal (21). The latter two pathways leading to ERK activation may explain the residual activity in cells inhibited with SPD-D (23). In cells treated with a relatively high dose of bombesin (50 nM), SPD-D stimulates the activation of Raf-1 and ERK to a level beyond that stimulated by bombesin alone. This could be due to the release of βγ subunits from a G_12 family member. Activation of Raf and ERK through a G_q pathway was eliminated as a possibility because this activation was not pertussis toxin-sensitive. SPD-D by itself does not stimulate ERK activity or Raf activity in these fibroblasts. This suggests that whatever signals SPD-D can generate, they are not sufficient to activate the ERK pathway, although they apparently are sufficient to activate the JNK pathway. The input of signals unique to bombesin, such as those mediated by G_q, are required for ERK activation. Thus, the biased agonist hypothesis explains many of the data that have been difficult to reconcile in previous reports, demonstrating the usefulness of this theory.

One of the most important biological systems in which neuropeptides play a role is small cell lung cancer. The substance P derivatives have already been identified as a potential therapy for SCLC, but the mechanism by which they act is unknown. Once we determine the manner in which SPDs selectively activate different signaling pathways, we can use them to determine which signals lead to the cytotoxic response in SCLC. Knowing the signals responsible for SCLC cytotoxicity will allow us to manipulate them to enhance the therapeutic effectiveness of compounds of this type.

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[d-Arg\(^1\),d-Phe\(^5\),d-Trp\(^7,9\),Leu\(^{11}\)] Substance P Acts as a Biased Agonist toward Neuropeptide and Chemokine Receptors
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