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

Matthew B. Jarpe‡‡, Cindy Knall‡, Fiona M. Mitchell‡‡, Anne Mette Buhl‡, Emir Duzic‡, and Gary L. Johnson‡**

From the ‡Program in Molecular Signal Transduction, Division of Basic Sciences, National Jewish Medical Research Center, Denver, Colorado 80206, Invivos Pharmaceuticals, Tarrytown, New York 10591, and the **Department of Pharmacology, University of Colorado Health Sciences Center, Denver, Colorado 80262

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). 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, [D-Arg\(^1\),\(\text{d}-\text{Phe}^5\),\(\text{d}-\text{Trp}^{7,9}\),\(\text{Leu}^{11}\)] 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 Ca\(^{2+}\) 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 G\(_q\) (9). Heterologous expression of receptors and G proteins in Sf9 cells have shown functional coupling between one neuropeptide receptor, NK-1, and G\(_{15}\), G\(_{16}\), and G\(_{13}\) but not G\(_{12}\) (10). In addition to the mobilization of 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 and stress fibers (16, 17). The activation of JNK and the reorganization of the actin cytoskeleton are most likely mediated by members of the G\(_{13}\) family of G proteins, such as G\(_{14}\) and G\(_{13}\) (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 G family member (21).

The effects of SPD-D and similar compounds in 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 Ca\(^{2+}\) response through neuropeptide receptors are affected differently by SPD-D (22, 23). In Swiss 3T3 fibroblasts, SPD-D inhibits Ca\(^{2+}\) mobilization induced by bombesin (which acts on the human GRP receptor) with a maximal effect at 10 \(\mu\)M and an estimated IC\(_{50}\) of 2 \(\mu\)M. In contrast, SPD-D inhibits ERK-2 activation with a maximal effect at 50 \(\mu\)M and an estimated IC\(_{50}\) of 9 \(\mu\)M (22). In the presence of 3 \(\mu\)M bombesin, 10 \(\mu\)M SPD-D caused a nearly complete inhibition of the Ca\(^{2+}\) response and an approximately 50% inhibition of the ERK-2 response (22). In a similar manner, 50 \(\mu\)M SPD-D caused a 100% inhibition of the inositol 1,4,5-trisphosphate generation induced by 50 \(\mu\)M 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 cytoskeleton.
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 chemotactant 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\textsuperscript{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 organization in fibroblasts in the absence of Ca\textsuperscript{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 cytotoxic 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**—[Arg\textsubscript{1},D-Phe\textsubscript{5},D-Trp\textsubscript{7},Leu\textsubscript{9}] substance P, purchased from Bachem Bioscience, was stored in powder form at -20 °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-\beta-galactosidase antibody was purchased from Cappel. Recombinant human IL-8 was purchased from R&D Systems, recombinant C5a was from Sigma, and [Tyr\textsuperscript{4}] bombesin was from Peninsula Laboratories. \textsuperscript{125}I-Labeled C5a, IL-8, and bombesin were purchased from NEN Life Science Products.

**Cell Lines and Culture**—Swiss 3T3 fibroblasts were cultured in Dulbecco’s modified Engle’s medium with 10% fetal calf serum (DME-10% FCS) and split every 3 days. For each experiment, Swiss 3T3 cells were split 1:10 and grown for 1 day in DME-10% FCS and then starved for 1 day in DME-0.1% calf serum prior to the experiment.

**Calcium Measurement in Rat 1A Cells**—Cells were transfected with the empty vector (pCMV5) or with pCMV5-GRPR using Pfx-3 transfection reagent (InVitrogen). After 1 day of recovery, the cells were plated with 0.1% calf serum into 12-well plates and starved for 1 day. The cells were then loaded with 1 mM Fluo-3 AM (Molecular Probes) for 30 min at room temperature in HEPES-buffered DME-0.1% calf serum. The cells were washed and intracellular calcium was determined by imaging on a laser confocal microscope. The images were processed using COMOS software (Bio-Rad). The brightness of each 40 field of approximately 10 cells was determined and divided by the baseline value to give the relative Fluo-3 fluorescence.

**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 \textmu M SPD-D was used in previous studies to inhibit inositol 1,4,5-triphosphate generation and ERK activation by bombesin (23). Rather than inhibiting bombesin-stimulated JNK activation, 50 \textmu M SPD-D caused a similar activation of JNK (Fig. 1A), which reached a maximum at 5 min (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 \mu M.

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 neuropeptides (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\textsuperscript{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 \mu M. 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 calciumion 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 \textmu M bombesin caused a rapid Ca\textsuperscript{2+} flux. SPD-D did not stimulate Ca\textsuperscript{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 DME-10% FCS, and the actin cytoskeleton was stained with rhodamine-phalloidin at a 1:40 dilution. The slides were mounted in \alpha-nylendiamine mounting medium and visualized on a digital confocal microscope. The images were deconvolved using Slidebook software (Intelligent Imaging Innovations, Inc.).
Fig. 1. **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. JNK 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 JNK 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.

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 G₁₂ family of G subunits (9), whereas it is likely that JNK activation is mediated by G₁₂ family members (31). SPD-D therefore acts as a biased agonist, activating G₁₂ and subsequent signaling components, without activating G₁₅ 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 actin microspikes and filopodia in Swiss 3T3 cells (15). Like JNK activation, this cytoskeletal reorganization is mediated by the G₁₂ family of G proteins (19). To further test the idea that SPD-D can activate G₁₂ in the absence of G₁₅ 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...
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, formylmethionylleucylphenylalanine, 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 recep-

Biased Agonist Properties of a Substance P Derivative
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^{2+} Mobilization in Human Neutrophils—IL-8 and substance P also cause a mobilization of Ca^{2+} in neutrophils. The mechanism for this Ca^{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^{2+} flux is the activation of a G_{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^{2+} from intracellular stores in human neutrophils (Fig. 7B). This Ca^{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^{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^{2+} response, or that the chemotaxis assay is much more sensitive than the Ca^{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^{2+} response to IL-8 and substance P is sensitive to pertussis toxin (36), suggesting the involvement of a G_{i} family member. The SPD-D-induced Ca^{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_{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^{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-
Biased Agonist Properties of a Substance P Derivative

FIG. 7. SPD-D induces a calcium mobilization in human neutrophils. Neutrophils were preloaded with Indo I AM, and Ca\(^{2+}\) release was monitored by flow cytometry. Cells were treated with 1.5 μM SPD-D (A) or 50 μM SPD-D (B). The ratio of the emission at 390 nm over the emission at 490 nm was determined and is presented in arbitrary units. Traces are representative of four independent experiments.

FIG. 8. Biased agonist hypothesis for the activation of receptors by SPD-D. A receptor, \(R\), is capable of spontaneously cycling between two active conformational states, \(R^{*1}\) and \(R^{*2}\), and one energetically favored inactive state, \(R\). \(R^{*1}\) is the conformation that activates \(G_{q}\), whereas \(R^{*2}\) is the state that activates \(G_{12}\). An agonist (\(A\)) binds to and stabilizes both activation states. An antagonist (\(AA\)) binds to all three states, competing with the agonist, and it may or may not stabilize the active states. If an antagonist stabilizes the active conformations of the receptor, it is termed a partial agonist. An inverse agonist (\(IA\)) binds to and further stabilizes the inactive state of the receptor. A biased agonist (\(BA\)) binds preferentially to one of the active states and stabilizes it, thus initiating one set of signal transduction events downstream of the receptor without activating others.

protein; \(R^{*1}\) can activate \(G_{q}\) for example, and \(R^{*2}\) can activate \(G_{12}\). 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 \(G_{q}\) through the C1a receptor, whereas it takes up to 10 nM calcitonin to activate \(G_{q}\) through this same receptor (30). There are two possible explanations for this difference. Either the \(G_{q}\) 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 \(G_{q}\) with a greater affinity than it stabilizes the form that activates \(G_{q}\). If the second case is true, there should be agonists that preferentially activate \(G_{q}\). To date, no examples of such a reversal of agonist efficacy have been found 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 \(R^{*2}\) over \(R^{*1}\), resulting in a receptor configuration that activates \(G_{12}\) but not \(G_{q}\). 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 \(G_{q}\) and \(G_{12}\) but not to \(G_{q}\). Therefore, SPD-D prefers the states of the receptor that can activate \(G_{q}\) and \(G_{12}\). 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

Biased agonism is a unique property of certain compounds that can selectively activate one G protein-coupled receptor over another, even when both receptors are present. This selective activation can be achieved in the absence of ligand, and it can occur between different classes of G proteins, including Gq, Gs, and Gi.

The concept of biased agonism is rooted in the idea that some compounds can preferentially activate a single G protein over others, leading to a biased response. This selectivity can be observed in various biological systems, including the regulation of intracellular calcium levels, inositol triphosphate production, and phospholipase C activation.

One of the most important biological systems in which neuropeptide signaling has been studied is the neuroendocrine system. Neuropeptides such as bombesin and vasoactive intestinal peptide (VIP) can activate multiple G protein-coupled receptors, leading to complex signaling cascades.

The original goal of this research was to resolve the mechanism for the differential inhibition of neuropeptide-induced Ca2+ responses and ERK activation. In Swiss 3T3 fibroblasts, Gq, Gs, and Gi proteins of all four major classes are present. Neuropeptide receptors have been shown to directly couple to three of these classes, Gq, Gs, and Gi. The activation of Ca2+ mobilization proceeds mainly through Gq, and JNK activation by neuropeptides may proceed through Gi.

The activation of ERKs by neuropeptides proceeds by a complex mechanism, with potential roles for PKC (20), βy 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 βy subunits from a Gi family member.

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.

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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Biased Agonist Properties of a Substance P Derivative

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