Substance P analogues including [D-Arg1,D-Trp5,7,9,Leu11]SP are broad spectrum neuropeptide antagonists and potential anticancer agents, but their mechanism of action is not fully understood. Here, we examined the mechanism of action of [D-Arg1,D-Trp5,7,9,Leu11]SP as an inhibitor of G protein-coupled receptor (GPCR)-mediated signal transduction and cellular DNA synthesis in Swiss 3T3 cells. Addition of [D-Arg1,D-Trp5,7,9,Leu11]SP, at 10 μM, caused a striking rightward shift in the dose-response curves of DNA synthesis induced by bombesin, bradykinin, or vasopressin and markedly inhibited the activation of p42mapk (ERK-2) and p44mapk (ERK-1) induced by these GPCR agonists. In addition, this SP analogue also prevented the protein kinase C-dependent activation of protein kinase D induced by these agonists. [D-Arg1,D-Trp5,7,9,Leu11]SP, at a concentration (10 μM) that inhibited these Gα-mediated events, also prevented GPCR agonist-induced responses mediated through the G proteins of the G12 subfamily. These include bombesin-induced assembly of focal adhesions, formation of parallel arrays of actin stress fibers, increase in tyrosine phosphorylation of focal adhesion protein paxillin and markedly inhibited the activation of p42mapk (ERK-2) and p44mapk (ERK-1) induced by these GPCR agonists.
Several models have been proposed to explain the molecular mechanism(s) by which agonists promote GPCR activation (see Ref. 42 for review). The widely used conformational selection model envisions that GPCRs cycle spontaneously (i.e. in the absence of ligand) between different conformational states: the inactive (R) and the active (R*) state (42). Agonists are thought to stabilize the active conformation of the receptor thereby increasing the probability for receptor-mediated activation of G protein (42). Receptors capable of activating two different G proteins (e.g. the bombesin/GRP receptor that interacts with Gq and G12) have been proposed to exist in three conformational states as follows: an inactive state that is favored energetically in the absence of agonist (R), a state that activates G12 (R*) and a state that activates Gq (R**). In this context, Jarpe and collaborators (43) proposed recently that SP analogues act in a novel manner, stabilizing the active conformation of the bombesin/GRP receptor that interacts with G12 and proportionally reducing the receptor population that interacts with Gq. In the framework of this model, SP analogues would act as agonists for G12-mediated events and as antagonists for Gq-mediated events. It was further suggested that the inhibitory effect of broad spectrum SP antagonists on neuropeptide-stimulated cell proliferation could result, at least in part, from the disruption of the coordinated signaling that normally emanates from GPCRs. In support of this model, Jarpe et al. (43) demonstrated that [D-Arg1,D-Trp5,7,9,Leu11]SP, a less potent analogue of [D-Arg1,D-Trp5,7,9,Leu11]SP induced actin reorganization and assembly of focal adhesions. Our previous results, however, lead us to conclude that [D-Arg1,D-Trp5,7,9,Leu11]SP coordinates inhibits signal transduction pathways activated by bombesin and other neuropeptides, but focal adhesion assembly and actin remodeling were not evaluated (20, 39).

The experiments presented here were designed to examine the mechanism of action of [D-Arg1,D-Trp5,7,9,Leu11]SP as an inhibitor of neuropeptide-mediated signal transduction and cellular DNA synthesis. Specifically, we examined whether this synthetic peptide antagonist, at concentrations that selectively inhibit neuropeptide-induced DNA synthesis and ERK activation, acts as an agonist of G12-mediated events including assembly of focal adhesions, formation of stress fibers, and tyrosine phosphorylation of the focal adhesion proteins FAK, p130Cas, and paxillin. On the basis of the results presented here, we conclude that [D-Arg1,D-Trp5,7,9,Leu11]SP acts as a mitogenic antagonist of neuropeptide GPCRs blocking signal transduction via both Gq and G12.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Stock cultures of Swiss 3T3 cells were maintained in DMEM, supplemented with 10% fetal bovine serum in a humidified atmosphere containing 10% CO2 and 90% air at 37 °C. For experimental purposes, Swiss 3T3 cells were plated in 100-mm dishes at 6 × 105 cells/dish or 35-mm dishes at 1 × 105 cells/dish in DMEM containing 10% fetal bovine serum and used after 6–8 days when the cells were confluent and quiescent.

**Immunoprecipitation**—Quiescent cultures of Swiss 3T3 cells (1–2 × 106 cells) were washed twice with DMEM and incubated for 5 min at 37 °C with or without [D-Arg1,D-Trp5,7,9,Leu11]SP. The cultures were incubated with various agonists as indicated and incubated for a further 5 min at 37 °C. The stimulation was terminated on ice by aspirating the medium and solubilizing the cells with 200 μl of 2× SDS-PAGE sample buffer. The samples were boiled for 10 min, resolved by 10% SDS-PAGE, and transferred to Immobilon-P membranes. Western Blotting—After SDS-PAGE, proteins were transferred to Immobilon-P membranes. After transfer, membranes were blocked using 5% nonfat dried milk in phosphate-buffered saline, pH 7.2, and incubated for at least 2 h at 22 °C with the desired antibodies diluted in phosphate-buffered saline, pH 7.2, containing 3% nonfat dried milk. Bound primary antibodies to immuno reactive bands were visualized by enhanced chemiluminescence (ECL) detection with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies.

**Assay of p42/44 MAPK (ERK-2) and p44/42 MAPK (ERK-1)** Activation—Quiescent cultures of Swiss 3T3 cells grown on 35-mm dishes were washed twice with DMEM and incubated for 5 min at 37 °C with or without [D-Arg1,D-Trp5,7,9,Leu11]SP, as indicated. The cultures were washed twice with DMEM and incubated for 5 min at 37 °C. The stimulation was terminated on ice by aspirating the medium and solubilizing the cells with 200 μl of 2× SDS-PAGE sample buffer. The samples were boiled for 10 min, resolved by 10% SDS-PAGE, and transferred to Immobilon-P membranes. Activation of p42/44MAPK and p44/42MAPK occurs through phosphorylation of specific threonine and tyrosine residues (45) resulting in slower migrating forms in SDS-PAGE gels. These activated forms were monitored by using a specific anti-phospho-p44/p42 MAPK-mAb (New England Biolabs) that recognizes only the activated forms phosphorylated on Thr-202 and Tyr-204.

**Kinase Assay of PKD**—The kinase activity of PKD was determined in an *in vitro* kinase assay by mixing 20 μl of immunocomplexes with 10 μl of a phosphorylation mixture containing (final concentration) 100 μM [γ-32P]ATP (specific activity 400–600 cpm/pmol), 30 mM Tris-HCl, pH 7.4, 10 mM MgCl2, and 1 mM DTT. After 10 min of incubation at 30 °C, the reaction was stopped by washing with 1 ml of kinase buffer and then adding 200 μl equal volume of 2× SDS-PAGE sample buffer (200 μl Tris-HCl, pH 6.8, 2 mM EDTA, 0.1 mM Na3VO4, 6% SDS, 10% glycerol, and 4% 2-mercaptoethanol) followed by SDS-PAGE analysis. The gels were dried, and the 110-kDa radioactive band corresponding to autophosphorylated PKD (44) was visualized by autoradiography.

**DNA Synthesis Measurements**—Confluent and quiescent cultures of Swiss 3T3 cells were washed twice with DMEM and incubated with DMEM/Waymouth’s medium (1:1, v/v) containing 10% FBS and incubated in 5% trichloroacetic acid at 4 °C for 20 min to remove acid-soluble radioactivity, washed with ethanol, and solubilized in 1 ml of 2% NaOH, and 0.1 mM NaOH. The acid-insoluble radioactivity was determined by scintillation counting in 6 ml of Beckman ReadySafe.

**Immunostaining of Cells**—Swiss 3T3 cells in 35-mm dishes were washed with serum-free DMEM and treated with agonists and/or inhibitors at 37 °C as indicated. For staining of actin, cells were washed twice with PBS, fixed in 4% paraformaldehyde in PBS for 10 min at room temperature, permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature, and blocked with 10% fetal bovine serum in PBS. The cells were then incubated with TRITC-conjugated phalloidin (0.25 μg/ml) in PBS for 10 min at room temperature and washed five times with PBS. In order to visualize focal adhesions, Swiss 3T3 cells were fixed, permeabilized as described above, and incubated with anti-vinculin mAb (dilution 1:100) for 2 h at room temperature. Cells were subsequently washed three times with PBS and then incubated with FITC-labeled anti-mouse IgG as second antibody at a dilution of 1:100 for another 30 min at room temperature. Immunofluorescence was visualized in both cases using a Zeiss immunofluorescence microscope. Images were collected with a Zeiss Confocal microscope.

**Materials**—Bombesin, bradykinin, vasopressin, Posturella multiformis toxin, anti-vinculin mAb, and TRITC-conjugated phalloidin were obtained from Sigma. ECL reagents were from Amersham Pharmacia Biotech. [D-Arg1,D-Trp5,7,9,Leu11]SP and [D-Arg1,D-Phe1,D-Trp5,7,9,Leu11]SP were obtained from Bachem. FAK polyclonal Ab-C-20, Src polyclonal Ab, and Tyr(P) monoclonal Ab PY20 were from Santa Cruz Biotechnol...
Fig. 1. [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP preferentially inhibits the mitogenic activity of neuropeptide-activated GPCRs. A. bombesin, bradykinin, and vasopressin dose-response relationships in the absence (●) and presence (○) of [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP. Confluent and quiescent cultures of Swiss 3T3 cells were washed and incubated at 37 °C in 2 ml of DMEM/Waymouth’s medium containing 1 μCi/ml [³H]thymidine and either increasing concentrations of bombesin (with 100 ng/ml insulin) or bradykinin (with 500 ng/ml insulin) or vasopressin (with 100 ng/ml insulin) either in the absence (●) or presence (○) of 10 μM [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP. After 40 h, DNA synthesis was assessed by measuring the [³H]thymidine incorporated into acid-precipitable material. Results are expressed as a percentage of the incorporation induced by the highest concentration of agonists, and data are shown as the mean ± S.E. (n = 3).

RESULTS

[D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP Selectively Inhibits Neuropeptide-induced DNA Synthesis—To examine whether [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP preferentially inhibits the mitogenic activity of neuropeptide-activated GPCRs, quiescent cultures of Swiss 3T3 cells, arrested in the G0 phase of the cell cycle, were transferred to serum-free media supplemented with [³H]thymidine and increasing concentrations of EGF (with 100 ng/ml insulin), PDGF, PDBu, or PGF2α dose-response relationships in the absence (●) and presence (○) of [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP. Confluent and quiescent cultures of Swiss 3T3 cells were washed and incubated at 37 °C in 2 ml of DMEM/Waymouth’s medium containing 1 μCi/ml [³H]thymidine and increasing concentrations of EGF (with 100 ng/ml insulin), PDGF, PDBu (with 100 ng/ml insulin), or PGF2α (with 100 ng/ml insulin) either in the absence (●) or presence (○) of 10 μM [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]Substance P. After 40 h, DNA synthesis was assessed by measuring the [³H]thymidine incorporated into acid-precipitable material. Results are expressed as a percentage of the incorporation induced by the highest concentration of agonists, and data are shown as the mean ± S.E. (n = 3).

Addition of [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP caused a striking rightward shift in the dose-response curves of DNA synthesis induced by bombesin, bradykinin, and vasopressin which act via distinct GPCRs (Fig. 1). The inhibitory effect was reversed at the higher concentrations of each peptide agonist (Fig. 1A), suggesting that [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP inhibits the mitogenic activity of neuropeptide-activated GPCRs in a competitive fashion.

In contrast, [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP did not interfere with the mitogenic effect induced via EGFR or PDGFR tyrosine kinase receptors or by pharmacological agents that directly activate signal transduction pathways bypassing receptors including the biologically active phorbol ester phorbol 12,13-dibutyrate (PDBu), a direct activator of the classic and novel isoforms of PKC (Fig. 1B), or the P. multocida toxin, a potent mitogen that activates Gαq (results not shown). Similarly, exposure to [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP did not affect DNA synthesis stimulated by PGF2α, which acts through a GPCR coupled to Gαq (49, 50). Taken together, the results presented in Fig. 1 substantiate the notion that [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP selectively targets GPCRs for mitogenic neuropeptides.

[D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP Prevents Gαq-mediated PKD Activation—In order to test further the selectivity of [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP as an inhibitor of neuropeptide-mediated mitogenic signals, we also determined the effect of this synthetic peptide on early activation of p42mapk (ERK-2) and p44mapk (ERK-1) induced by multiple agonists. To examine ERK activation, lysates of Swiss 3T3 cells incubated for 5 min in the absence or in the presence of 10 μM [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP and subsequently challenged with multiple stimuli were analyzed by immunoblotting using a site-specific antibody that recognizes the dual phosphorylated (active) forms of ERK-1 and ERK-2.

As shown in Fig. 2, ERK activation induced by stimulation with 1 nM bombesin, 5 nM vasopressin, or 5 nM bradykinin was dramatically reduced by exposure of the cells to 10 μM [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP. In contrast, ERK activation induced by either PDGF (2.5 ng/ml) or EGF (2.5 ng/ml) was not affected by addition of [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP, at an identical concentration. Similarly, prior exposure to [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP did not affect ERK activation via direct stimulation of PKC with PDBu or through the Gαq-coupled receptor for PGF2α.

[D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP Prevents Gαq-mediated PKD Activation—PKD/PKCμ is a serine/threonine protein kinase (51, 52) with structural, enzymological, and regulatory properties.
Fig. 2. [d-Arg¹,d-Trp⁵,⁷,⁹,Leu¹¹]Substance P preferentially inhibits the ERK activation of neuropeptide-activated GPCRs. A, inhibition of ERK activation induced by bombesin, bradykinin, and vasopressin by [d-Arg¹,d-Trp⁵,⁷,⁹,Leu¹¹]Substance P. Confluent and quiescent Swiss 3T3 cells were washed twice with DMEM and incubated for 5 min with 10 μM [d-Arg¹,d-Trp⁵,⁷,⁹,Leu¹¹]Substance P. Cells were subsequently challenged with bombesin (1 nM), bradykinin (5 nM), or vasopressin (5 nM) for 5 min. B–D, [d-Arg¹,d-Trp⁵,⁷,⁹,Leu¹¹]Substance P does not inhibit ERK activation induced by EGF, PDGF, PDBu, and PGF₂α. Confluent and quiescent Swiss 3T3 cells were washed twice with DMEM and incubated for 5 min with 10 μM [d-Arg¹,d-Trp⁵,⁷,⁹,Leu¹¹]Substance P. Cells were then stimulated with EGF (2.5 ng/ml), PDGF (1 ng/ml), PDBu (at the indicated concentrations), or PGF₂α (at the indicated concentrations) for 5 min. Cells were then lysed in 2× SDS-PAGE sample buffer and analyzed by SDS-PAGE and immunoblotting with phospho-ERK antibody as described under “Experimental Procedures.” The results presented here are typical of three independent experiments.

Fig. 3. [d-Arg¹,d-Trp⁵,⁷,⁹,Leu¹¹]SP inhibits PKD activation by bombesin, bradykinin, and vasopressin. A, confluent and quiescent Swiss 3T3 cells were washed twice with DMEM and incubated for 5 min with increasing concentrations of [d-Arg¹,d-Trp⁵,⁷,⁹,Leu¹¹]SP (SP analogue), as indicated. The results are expressed as a percentage of the maximal activation obtained with bombesin in the absence of [d-Arg¹,d-Trp⁵,⁷,⁹,Leu¹¹]SP. B, the experimental conditions were identical to those described in A except that the cells were stimulated with either 10 nm bradykinin (BRK) or 10 nm vasopressin (VP) instead of bombesin.

Assembly of Focal Adhesions, and Formation of Stress Fibers—The preceding results indicate that [d-Arg¹,d-Trp⁵,⁷,⁹,Leu¹¹]SP selectively targets neuropeptide-mediated signals. Recently, it has been proposed that antagonists of this class act in a novel manner, stabilizing the active conformation of GPCRs that interact with G₁₂, thereby acting as an agonist for G₁₂-mediated events but as an antagonist for G₃₃-mediated events (43). In order to test this hypothesis, we determined the effect of [d-Arg¹,d-Trp⁵,⁷,⁹,Leu¹¹]SP on cellular responses stimulated by bombesin receptor via the α subunits of the G₁₂ subfamily.

Activated G₁₂ and G₃₃ (which comprise the G₁₂ subfamily) are known to induce Rho activation (15, 57–60) via recruitment and activation of a GDP/GTP exchange factor (61) and to promote Rho-dependent stress fiber formation and focal adhesion assembly in Swiss 3T3 cells (15, 62). Consequently, if [d-Arg¹,d-Trp⁵,⁷,⁹,Leu¹¹]SP acts as a selective G₁₂ agonist, it should induce focal adhesion assembly and stress fiber formation at concentrations that inhibit mitogenic signaling in Swiss 3T3 cells. To test this prediction, quiescent cultures of these cells were preincubated in the absence or in the presence of 10 μM [d-Arg¹,d-Trp⁵,⁷,⁹,Leu¹¹]SP, challenged with or without bombesin and then fixed. Focal adhesions were visualized by staining with anti-vinculin mAb (Fig. 4), and the organization of the actin cytoskeleton was revealed with TRITC-phalloidin (Fig. 5).

Quiescent cultures of Swiss 3T3 cells exhibited only very few focal adhesions. As expected, stimulation with 1 nm bombesin induced a dramatic increase in the assembly of well-defined, pear-shaped focal adhesions (Fig. 4) and in the formation of parallel arrays of actin stress fibers (Fig. 5). In contrast, pretreatment of the cells with [d-Arg¹,d-Trp⁵,⁷,⁹,Leu¹¹]SP at 10 μM, a concentration that markedly inhibited neuropeptide-mediated G₁₂ signaling and DNA synthesis (Figs. 1–3), did not induce a significant increase in the formation of either focal adhesions (Fig. 4) or stress fibers (Fig. 5). The salient feature of these experiments is that pretreatment of the cells with 10 μM
FIG. 4. [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP inhibits assembly of focal adhesions in Swiss 3T3 cells induced by bombesin. Confluent and quiescent Swiss 3T3 cells were washed and preincubated without (−) or with (SP analogue) 10 μM [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹] Substance P for 5 min at 37 °C and then challenged for a further 10 min either in the absence (−) or the presence (Bom) of 1 nM bombesin. Cells were then washed, fixed in 4% paraformaldehyde, and permeabilized with 0.2% Triton X-100. Focal adhesions were visualized by staining with vinculin mAb followed by FITC-labeled anti-mouse IgG and visualized using a Zeiss fluorescence microscope as described under “Experimental Procedures.” Results are typical of three independent experiments.

FIG. 5. [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP strikingly prevented the assembly of actin stress fibers in cells induced by bombesin. Confluent and quiescent Swiss 3T3 cells were washed and preincubated without (−) or with (SP analogue) 10 μM [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹] Substance P for 5 min at 37 °C and then incubated for a further 10 min either in the absence (−) or the presence (Bom) of 1 nM bombesin. Cells were then washed, fixed in 4% paraformaldehyde, and permeabilized with 0.2% Triton X-100. Focal adhesions were visualized by staining with TRITC-conjugated phalloidin (0.25 μg/ml) for 10 min and visualized using a Zeiss fluorescence microscope as described under “Experimental Procedures.” Results are typical of five independent experiments.

Inhibitory Effect of [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP on Additional G₁₂-mediated Events, Tyrosine Phosphorylation of FAK, Phosphorylation of FAK at Tyr-397, and the formation of the Src-FAK complex induced by bombesin. A, [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP inhibits tyrosine phosphorylation of FAK induced by bombesin. Confluent and quiescent cells were washed and incubated with 0.2% Triton X-100. Focal adhesions were visualized by staining with TRITC-conjugated phalloidin (0.25 μg/ml) for 10 min and visualized using a Zeiss fluorescence microscope as described under “Experimental Procedures.” Results shown are representative of four independent experiments. B, scanning densitometry. The results shown are the values (mean ± S.E.; n = 4) obtained by scanning densitometry, expressed as a percentage of the maximal FAK phosphorylation obtained with 1 nM bombesin in the absence of [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹] Substance P. All other experimental details are as described under “Experimental Procedures.” C, [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP inhibits phosphorylation of FAK at Tyr-397 and the formation of the Src-FAK complex induced by bombesin. Confluent and quiescent cells were washed and incubated without (−) or with (+) 10 μM [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹] Substance P (SP analogue). Cells were then stimulated with 1 nM bombesin (Bom) for 10 min at 37 °C and lysed. The lysates were immunoprecipitated with either an anti-FAK polyclonal antibody, and the immunoprecipitates were analyzed by Western blotting using an anti-Tyr(P) mAb as described under “Experimental Procedures.” Result shown are representative of four independent experiments.
promote FAK tyrosine phosphorylation but, in contrast, dramatically inhibited (90\%) the increase in the tyrosine phosphorylation of FAK induced by bombesin.

Extensive evidence indicates that FAK translocation to nascent focal adhesions promotes its autophosphorylation as a result of clustering and/or conformational changes. Because the major site of FAK autophosphorylation, Tyr-397, is a potential high affinity binding site for the SH2 domain of Src, the phosphorylation of this site in response to GPCR activation facilitates the formation of a FAK/Src signaling complex (63). In order to test further whether [d-Arg^1,d-Trp^5,7,9,Leu^11]SP acts as an antagonist for G_{12}-mediated events, we examined the effect of this synthetic peptide on bombesin-induced tyrosine phosphorylation of FAK at Tyr-397 and on Src-FAK complex formation which depends on the tyrosine phosphorylation of Tyr-397. The results, shown in Fig. 6C, indicate that treatment with 10 \mu M [d-Arg^1,d-Trp^5,7,9,Leu^11]SP neither induced a significant increase in the tyrosine phosphorylation of FAK at Tyr-397 nor promoted Src-FAK complex formation. In contrast, exposure to this SP analogue markedly inhibited the increase in the tyrosine phosphorylation of FAK at Tyr-397 and the formation of a complex between Src and FAK induced by bombesin.

In order to substantiate the results obtained with FAK, we examined the effect of [d-Arg^1,d-Trp^5,7,9,Leu^11]SP on bombesin-induced tyrosine phosphorylation of the adaptor proteins p130^{Cas} and paxillin. As illustrated by Fig. 7, addition of [d-Arg^1,

\textbf{D - Trp^5,7,9,Leu^11}]SP at 10 \mu M did not induce tyrosine phosphorylation of either p130^{Cas} or paxillin. In contrast, this SP analogue markedly inhibited the increase in the tyrosine phosphorylation of p130^{Cas} and paxillin induced by bombesin. Taken together, our results support the conclusion that [d-Arg^1,d-Trp^5,7,9,Leu^11]SP (at 10 \mu M) acts as an antagonist that coordinately prevents GPCR-induced events mediated by either G_5 or G_{12}.

[d-Arg^1,d-Phe^5,d-Trp^5,7,9,Leu^11]SP Inhibits Focal Adhesion Assembly and Tyrosine Phosphorylation of FAK and Paxillin Induced by Bombesin—The results presented here appear to contrast with those reported previously by Jarpe et al. (43) who demonstrated that [d-Arg^1,d-Phe^5,d-Trp^5,7,9,Leu^11]SP, a less potent analogue of [d-Arg^1,d-Trp^5,7,9,Leu^11]SP, induced actin reorganization and focal adhesion assembly. However, [d-Arg^1,d-Phe^5,d-Trp^5,7,9,Leu^11]SP promoted these responses at concentrations higher than those used in the present study, and the possibility that this SP analogue, at a lower concentration, could also inhibit G_{12}-mediated events, as shown here with [d-Arg^1,d-Trp^5,7,9,Leu^11]SP, was not explored. In an effort to reconcile these discrepancies, we determined the effect of [d-Arg^1,d-Phe^5,d-Trp^5,7,9,Leu^11]SP on bombesin-induced focal adhesion assembly and tyrosine phosphorylation of FAK and paxillin.

As shown in Fig. 8, treatment with 20 \mu M [d-Arg^1,d-Phe^5,d-Trp^5,7,9,Leu^11]SP neither promoted focal adhesion assembly nor induced a significant increase in the tyrosine phosphorylation of FAK and paxillin. In contrast, exposure to this SP analogue markedly increased the increase in focal adhesion assembly and tyrosine phosphorylation of FAK and paxillin induced by bombesin stimulation (Fig. 8). Thus, [d-Arg^1,d-Phe^5,d-Trp^5,7,9,Leu^11]SP, like [d-Arg^1,d-Trp^5,7,9,Leu^11]SP, inhibited focal adhesion assembly and tyrosine phosphorylation of FAK and paxillin.

[d-Arg^1,d-Trp^5,7,9,Leu^11]SP Stimulates Morphological Responses and Tyrosine Phosphorylation of FAK and Paxillin at High Concentrations—We also examined the effect of [d-Arg^1,d-Trp^5,7,9,Leu^11]SP at focal adhesion assembly and tyrosine phosphorylation of FAK and paxillin at concentrations higher than 10 \mu M. As shown in Fig. 9, exposure of Swiss 3T3 cells to 50 \mu M [d-Arg^1,d-Trp^5,7,9,Leu^11]SP promoted the assembly of only few focal adhesions and caused actin cytoskeletal changes that were not equivalent to the parallel arrays of stress fibers induced by bombesin (results not shown). Furthermore, addition of [d-Arg^1,d-Trp^5,7,9,Leu^11]SP at 50 \mu M induced a low level of tyrosine phosphorylation of FAK and paxillin as compared with the effect induced by bombesin in parallel cultures. Interestingly, exposure of the cells to [d-Arg^1,d-Trp^5,7,9,Leu^11]SP at 50 \mu M markedly reduced the assembly of focal adhesions and the increase in tyrosine phosphorylation of focal adhesion proteins induced by bombesin. We conclude that [d-Arg^1,d-Trp^5,7,9,Leu^11]SP acts as an antagonist of neuropeptide GPCRs that signal via both G_5 and G_{12}, but at higher concentrations, this synthetic peptide induces additional effects that lead to limited assembly of focal adhesions and to a low level of tyrosine phosphorylation of focal adhesion proteins.

\textbf{DISCUSSION}

SP analogues including [d-Arg^1,d-Phe^5,d-Trp^5,7,9,Leu^11]SP and [Arg^6,d-Trp^5,7,9,MePhe^8]SP (6–11) are an interesting class of agents that inhibit the activation of signal transduction pathways and the mitogenic action of a range of neuropeptides structurally unrelated to SP. These synthetic peptides also inhibit the proliferation of human cancer cells both \textit{in vitro} and in nude mice and have entered in phase I clinical trials. Consequently, the mechanism of action of these agents is attracting considerable interest.

Jarpe and collaborators (43) proposed recently that broad
It is well established that GPCR-induced increases in tyrosine phosphorylation of FAK, paxillin, and p130Cas are down-stream of Rho activation, stress fiber formation, and focal adhesion assembly (64–70). Activated G_{12}, and G_{13} are known to induce Rho activation (15, 57–59) via recruitment and activation of a GDP/GTP exchange factor (61). Furthermore, activated G_{12} and G_{13} stimulate tyrosine phosphorylation of FAK, paxillin, and p130Cas via Rho (16). Taken together, these findings indicate the existence of a signal transduction pathway whereby agonist occupation of GPCRs activates G_{12} and/or G_{13}, leading to Rho-dependent formation of actin stress fibers and assembly of focal adhesions resulting in the recruitment of FAK to focal adhesions and in the tyrosine phosphorylation of FAK, paxillin, and p130Cas. Therefore, we examined whether [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP promotes the selective activation of this pathway in Swiss 3T3 cells challenged with or without bombesin. Confluent and quiescent Swiss 3T3 cells were washed and preincubated without (−) or with 1 nM bombesin (Bom). Cells were then washed, fixed in 4% paraformaldehyde, and permeabilized with 0.2% Triton X-100. Focal adhesions were visualized by staining with vinculin mAb followed by FITC-labeled anti-mouse IgG and visualized using a Zeiss fluorescence microscope as described under “Experimental Procedures.” Results are typical of three independent experiments. B, effect of 50 μM [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹] Substance P on tyrosine phosphorylation of FAK and paxillin in Swiss 3T3 cells challenged with or without bombesin. Confluent and quiescent cells were washed and incubated for 5 min at 37°C without (−) or with (+) 50 μM [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹] Substance P (SP analogue). Cells were then stimulated with 1 nM bombesin for 10 min at 37°C and lysed. The lysates were then immunoprecipitated with either anti-FAK polyclonal antibody or paxillin mAb, and the immunoprecipitates were analyzed by Western blotting using an anti-Tyr(P) mAb described under “Experimental Procedures.” Results shown are representative of three independent experiments.

FIG. 9. Effect of high concentrations of [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹] Substance P on assembly of focal adhesions and tyrosine phosphorylation of focal adhesion proteins. A, effect of high concentrations of [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹] Substance P on assembly of focal adhesions in Swiss 3T3 cells challenged with or without bombesin. Confluent and quiescent Swiss 3T3 cells were washed and preincubated without (−) or with (50 μM [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹] Substance P for 5 min at 37°C. The cells were then incubated for a further 10 min either in the absence (−) or the presence of 1 nM bombesin (Bom). Cells were then washed, fixed in 4% paraformaldehyde, and permeabilized with 0.2% Triton X-100. Focal adhesions were visualized by staining with vinculin mAb followed by FITC-labeled anti-mouse IgG and visualized using a Zeiss fluorescence microscope as described under “Experimental Procedures.” Results are typical of three independent experiments. B, effect of 50 μM [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹] Substance P on tyrosine phosphorylation of FAK and paxillin in Swiss 3T3 cells challenged with or without bombesin. Confluent and quiescent cells were washed and incubated for 5 min at 37°C without (−) or with (+) 50 μM [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹] Substance P (SP analogue). Cells were then stimulated with 1 nM bombesin for 10 min at 37°C and lysed. The lysates were then immunoprecipitated with either anti-FAK polyclonal antibody or paxillin mAb, and the immunoprecipitates were analyzed by Western blotting using an anti-Tyr(P) mAb described under “Experimental Procedures.” Results shown are representative of three independent experiments.
way, we examined in the present study the effects of [D-Arg₁,D-Trp⁵,⁷,⁹,Leu¹¹]SP on the assembly of focal adhesions, the formation of actin stress fibers, and the tyrosine phosphorylation of FAK, paxillin, and p130Cas.

Our results demonstrate that pretreatment of Swiss 3T3 cells with 10 μM [D-Arg₁,D-Trp⁵,⁷,⁹,Leu¹¹]SP, a concentration that markedly inhibited neuropeptide-mediated G₉ signaling and DNA synthesis, did not induce a significant increase in the formation of either focal adhesions or stress fibers. In contrast, pretreatment with [D-Arg₁,D-Trp⁵,⁷,⁹,Leu¹¹]SP strikingly prevented the assembly of focal adhesions and the increase in actin stress fibers induced by bombesin. Furthermore, our results also demonstrate that treatment with [D-Arg₁,D-Trp⁵,⁷,⁹,Leu¹¹]SP neither induced a significant increase in the overall tyrosine phosphorylation of FAK nor induced tyrosine phosphorylation of paxillin or p130Cas. A salient feature of the results presented here is that treatment with [D-Arg₁,D-Trp⁵,⁷,⁹,Leu¹¹]SP dramatically inhibited the increase in the tyrosine phosphorylation of these focal adhesion proteins induced by bombesin. Thus, [D-Arg₁,D-Trp⁵,⁷,⁹,Leu¹¹]SP, at a concentration that inhibits G₉ signaling and mitogenesis by neuropeptides, also prevents G₉-mediated events including focal adhesion assembly, stress fiber formation, and tyrosine phosphorylation of focal adhesion proteins.

Jarpe et al. (43) demonstrated that [D-Arg₁,D-Phe⁶,D-Trp⁷,⁹,Leu¹¹]SP, a less potent analogue of [D-Arg₁,D-Trp⁵,⁷,⁹,Leu¹¹]SP, induced actin reorganization and focal adhesion assembly at concentrations higher than that used in the present study. A possible explanation for the apparent discrepancy between our results obtained with [D-Arg₁,D-Trp⁵,⁷,⁹,Leu¹¹]SP and those with [D-Arg₁,D-Phe⁶,D-Trp⁷,⁹,Leu¹¹]SP is that a single amino acid change at position 5 from D-Phe⁶ to D-Trp⁶ is responsible for the drastic change in the properties of these antagonists. However, Jarpe et al. (43) did not examine the possibility that [D-Arg₁,D-Phe⁶,D-Trp⁷,⁹,Leu¹¹]SP, at a lower concentration, could also inhibit G₁₂-mediated events, as shown here with [D-Arg₁,D-Trp⁵,⁷,⁹,Leu¹¹]SP. In the present study, we show that [D-Arg₁,D-Phe⁶,D-Trp⁷,⁹,Leu¹¹]SP (at 20 μM) markedly inhibited bombesin-induced assembly of focal adhesions and tyrosine phosphorylation of FAK and paxillin. Furthermore, we could also demonstrate that exposure of Swiss 3T3 cells to 50 μM [D-Arg₁,D-Trp⁵,⁷,⁹,Leu¹¹]SP promoted cytoskeletal rearrangements, formation of some focal adhesions, and a low level of tyrosine phosphorylation of focal adhesion-associated proteins. These effects were much less prominent than those induced by bombesin in parallel cultures, and even at these concentrations, the SP analogue markedly reduced the assembly of focal adhesions and the tyrosine phosphorylation of focal adhesion proteins induced by bombesin.

In conclusion, [D-Arg₁,D-Trp⁵,⁷,⁹,Leu¹¹]SP acts as a broad spectrum mitogenic antagonist of neuropeptide GPCRs blocking signal transduction via both G₉ and G₁₂. However, at higher concentrations, this synthetic peptide induces additional effects that lead to a low level of focal adhesions and a low level of tyrosine phosphorylation of focal adhesion-associated proteins. These findings are not compatible with the hypothesis that [D-Arg₁,D-Trp⁵,⁷,⁹,Leu¹¹]SP stabilizes a bombesin receptor conformation that interacts selectively with G₁₂ but indicate that this SP analogue inhibits bombesin signaling mediated by both G₉ and G₁₂.

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