[d-Arg₁,d-Trp⁵,⁷,⁹,Leu¹¹]Substance P Coordinately and Reversibly Inhibits Bombesin- and Vasopressin-induced Signal Transduction Pathways in Swiss 3T3 Cells*

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The novel substance P (SP) analogue, [d-Arg₁,d-Trp⁵,⁷,⁹,Leu¹¹]SP like [d-Arg₁,d-Phe⁶,d-Trp⁷,⁹,Leu¹¹]SP inhibited DNA synthesis induced by bombesin, vasopressin, and bradykinin, but did not interfere with the mitogenic response induced by other growth factors or pharmacological agents in Swiss 3T3 cells. [d-Arg₁,d-Trp⁵,⁷,⁹,Leu¹¹]SP reversibly inhibited bombesin-induced DNA synthesis, causing a 6-fold greater rightward shift in the bombesin dose response than [d-Arg₁,d-Phe⁶,d-Trp⁷,⁹,Leu¹¹]SP at identical concentrations (10 μM). We found that the new, more potent, SP analogue coordinately and reversibly inhibited bombesin-induced Ca²⁺ mobilization and protein kinase C (PKC) and mitogen-activated protein (MAP) kinase activation. The dose-response curves for bombesin-induced Ca²⁺ mobilization and MAP kinase activation were similarly displaced (51% and 40-fold, respectively) by [d-Arg₁,d-Trp⁵,⁷,⁹,Leu¹¹]SP. In addition, [d-Arg₁,d-Trp⁵,⁷,⁹,MePhe⁸,Leu¹¹]SP reversibly inhibited bombesin-induced tyrosine phosphorylation of Mr 110,000–130,000 and 70,000–80,000 bands as well as p125 focal adhesion kinase. [d-Arg₁,d-Trp⁵,⁷,⁹,Leu¹¹]SP also reversibly and coordinately inhibited vasopressin-induced Ca²⁺ mobilization, PKC stimulation, MAP kinase activation, tyrosine phosphorylation, and DNA synthesis in Swiss 3T3 cells. Surprisingly, deletion of the terminal Leu of [d-Arg₁,d-Phe⁶,d-Trp⁷,⁹,Leu¹¹]SP to yield [d-Arg₁,d-Phe⁶,d-Trp⁷,⁹,SP]¹⁻¹⁰ resulted in a selective loss of inhibitory activity of this analogue against bombesin- but not vasopressin-stimulated DNA synthesis, Ca²⁺ mobilization, and MAP kinase activation. Collectively, these results suggest that SP analogues act at the receptor level to coordinately and reversibly antagonize bombesin- or vasopressin-induced signal transduction in Swiss 3T3 cells.

Neuropeptides act as potent cellular growth factors and have been implicated in a variety of normal and abnormal biological processes including development and tumorigenesis (1). In particular, bombesin, its mammalian homologue GRP,¹ and vasopressin are potent mitogens for quiescent Swiss 3T3 cells, a useful model for the elucidation of signal transduction pathways (2). The binding of bombesin or vasopressin to their receptors activates pertussis toxin-insensitive G proteins (3–7) of the Gₛ subfamily (8–10) which stimulate the PI₃,PLC-γ isoforms of PLC (11–16). This results in rapid hydrolysis of polyphosphoinositides, with the consequent mobilization of Ca²⁺ and activation of PKC (17–22) leading to phosphorylation of the prominent substrate 80K/MARCKS (4, 21, 23–26). Further downstream, bombesin stimulates MAP kinase activation via a PKC dependent pathway (27, 28) prior to stimulating DNA synthesis (1, 2). In addition, the binding of bombesin and vasopressin to their receptors has been shown to stimulate the rapid tyrosine phosphorylation of multiple substrates in Swiss 3T3 cells (29–35).

Interestingly, multiple neuropeptides, including bombesin and vasopressin, have also been implicated as autocrine and paracrine growth factors for small cell lung cancer (36), breast (37), and prostate cancer (38). Consequently, it may be useful to develop inhibitors of neuropeptide action, which have a broader spectrum than specific receptor antagonists (39).

SP analogues were initially synthesized to generate specific SP antagonists. Surprisingly, the synthetic SP analogues, [d-Arg₁,d-Phe⁶,d-Trp⁷,⁹,Leu¹¹]SP and [Arg₁,d-Trp⁷,⁹,MePhe⁸]SP (6–11) were found to inhibit the action of a broad range of neuropeptides structurally unrelated to SP, including bombesin- and vasopressin-stimulated DNA synthesis in Swiss 3T3 cells (40–44). In contrast, they did not inhibit mitogenesis stimulated by either vasoactive intestinal peptide, which induces cAMP accumulation via Gₛ, or platelet-derived growth factor, which signals through receptors with intrinsic tyrosine kinase activity (45). More recently, [d-Arg₁,d-Phe⁶,d-Trp⁷,⁹,Leu¹¹]SP and [Arg₁,d-Trp⁷,⁹,MePhe⁸]SP (6–11) have been shown to inhibit small cell lung cancer cell proliferation in liquid culture, soft agar, and as xenografts in nude mice (46–48). Despite their intriguing biological effects and potential importance as antiproliferative agents, the mechanism of action of SP analogues as broad spectrum inhibitors of neuropeptide-mediated signal transduction remains incompletely understood.

A recent report has proposed that [d-Arg₁,d-Phe⁶,d-Trp⁷,⁹,Leu¹¹]SP selectively uncouples PI₃,PLC-γ from the bombesin receptor (49). It was therefore suggested that the inhibitory effect of the SP analogues on cell proliferation could be attributed to disruption of the coordinated regulation of cyclase; mAb, monoclonal antibody; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; 80K/MARCKS, 80K/myristoylated alanine-rich protein kinase C substrate; PI₄,5-P₂, phosphatidylinositol 4,5-bisphosphate; PLC-β, specific phospholipase C-β isoform; PKC, protein kinase C; PAGE, polyacrylamide gel electrophoresis; SP, substance P; Hepp, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid.

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¹ The abbreviations used are: GRP, gastrin-releasing peptide; Tyr(P), phosphotyrosine; DMEM, Dulbecco’s modified Eagle’s medium; EGF, epidermal growth factor; FAK, focal adhesion kinase; Fura-2/AM, Fura 2-tetraacetoxymethyl ester; G protein, guanine nucleotide-binding regulatory protein; Gₛ, designation of class of heterotrimeric proteins; Gₛₐ, a heterotrimeric G protein that mediates stimulation of adenylate"
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**Experimental Procedures**

**Cell Culture**—Stock cultures of Swiss 3T3 fibroblasts 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, cells were plated in 33- or 90-mm Nunc Petri dishes at 105 cells or 6 x 105 cells/dish, respectively, in DMEM containing 10% fetal bovine serum and used after 6–8 days when the cells were confluent and quiescent.

Assays of [3H]Thymidine Incorporation—Quiescent and confluent cells in 33-mm dishes were washed twice in DMEM and incubated at 37 °C in 2 ml of a 1:1 mixture of DMEM and Waymouth medium containing 1 μCi/ml [3H]thymidine with various additions as indicated. After 40 h, unless otherwise indicated, acid-precipitable material was measured as described previously (51).

Measurement of Intracellular Calcium—[Ca2+]i, was measured with the fluorescent Ca2+ indicator fura-2/AME using a modification of the procedure previously described (52). Quiescent cells in 90-mm dishes were washed twice in DMEM and then incubated at 37 °C for 10 min in 5 ml of DMEM with 1 μM fura-2 tetraacetoxymethyl ester. The dishes were washed three times in phosphate-buffered saline at 37 °C, and the cells were then suspended in 2 ml of electrolyte solution containing 120 mM NaCl, 5 mM KCl, 1.8 mM CaCl2, 0.9 mM MgCl2, 25 mM glucose, 16 mM Hepes, 6 mM Tris, and an amino acid mixture equivalent to DMEM (pH 7.2) by gentle scraping and transferred to a quartz cuvette. The suspension was stirred continuously and maintained at 37 °C. Various factors were added as indicated in the figure legends. Fluorescence was monitored in a Perkin-Elmer LS-5 luminescence spectrophotometer with an excitation wavelength of 336 nm and emission wavelength of 510 nm. [Ca2+]i with an excitation wavelength of 336 nm and emission wavelength of 345 nm was 220 nM for fura-2/AME (52).

**Assays of [32P] ATP Incorporation**—Quiescent cultures of Swiss 3T3 cells were treated with factors as described in the figure legends and lysed at 4 °C. Lysates were clarified by centrifugation at 15,000 × g for 20 min at 4 °C, and the supernatants were immunoprecipitated using the polyclonal anti-p42MAPK antibody together with protein A-agarose beads (40 μl, 1:1 slurry) for 2 h. Immune complexes were washed twice by centrifugation and washed once in lysis buffer and three times in kinase buffer (15 mM Tris-HCl, 15 mM MgCl2). The kinase reaction was performed by resuspending the pellet in 25 μl of kinase assay mixture containing kinase buffer, 1 mg/ml myelin basic protein-peptide (APRTPGGRR), 100 μCi ATP, 100 μCi/ml [γ-32P]ATP, and 200 μCi/mg microcin A1K. Incubations were performed for 10 min (linear assay conditions) at 30 °C and terminated by spotting 20 μl of the supernatant onto P81 chromatography paper (Whatman). Filters were washed four times, 5 min each, in 0.5% orthophosphoric acid, immersed in acetone, and dried before counting. The average radioactivity of two blank samples containing no immune complex was subtracted from the result of each sample. Results are expressed as a percentage of the maximum response obtained with either bombesin or vasoressin alone in the absence of inhibitors. The specific activity of [γ-32P]ATP used was 900–1200 cpm/pmole.

**SDS-PAGE**—Slab gel electrophoresis was performed essentially according to the method of Laemml (54). Specifically, the slab gels were 1.5 mm thick with 1.5 cm of a 4% acrylamide stacking gel and 12 cm of 8% or 10% acrylamide resolving gel. Samples (100 μl) were electrophoresed at 20 V for 30 min, then run overnight at 50 V, and finally at 150 V for 30 min before terminating the run. Gels for 80K/MARKCS experiments were fixed in 25% methanol, 10% acetic acid (v/v) prior to drying under vacuum for 2 at 80 °C. Radioactivity was detected at ~70 °C using Fuji x-ray film with exposure times of 12–72 h.

**Materials**—Bombesin, vasoressin, EGF, and Ig-agarose were obtained from Sigma. Protein A-agarose was from Boehringer Mannheim. Anti-Tyr(P) mAb (410 anti-Tyr(P)Mab) was from Upstate Biotechnology Inc., Lake Pladid, NY. The anti-p125Fak mAb for Western blotting was obtained from ABBINFIT Research Products Ltd., Nottingham, UK. The polyclonal anti-p42MAPK (anti-ERK-2) antibody raised against a COOH-terminal peptide (EETARFQPGYRS) was a generous gift from Dr. J. Van Lint (Katholieke Universiteit Leuven, Belgium). [125I]-Labeled sheep anti-mouse immunoglobulin G (15 μCi/mg), [125I]-labeled protein A (15 μCi/mg), carrier-free [32P]Pi (370 MBq/ml), γ-[32P]ATP (370 MBq/ml), and [3H]thymidine were from Amer-shar, UK. Fura 2/AME and forskolin were from Calbiochem. Methylsobutylinxanthine was purchased from Aldrich. [3H]Thymidine, and [3H]ATP (370 MBq/ml), [γ-32P]ATP (370 MBq/ml), and [3H]thymidine were from Amersham (UK). Fura 2/AME and forskolin were from Calbiochem. Methylsobutylinxanthine was purchased from Aldrich. [3H]Thymidine, and [3H]ATP (370 MBq/ml), [γ-32P]ATP (370 MBq/ml), and [3H]thymidine were from Amersham (UK). Fura 2/AME and forskolin were from Calbiochem.

**RESULTS**

Comparison of the Inhibitory Effect of [D-Arg1,D-Phe5,D-Trp7,9-Leu11]SP with [D-Arg1,D-Phe5,D-Trp7,9,Val11]SP on DNA Synthesis Induced by Various Agents—We have previously shown that substitution of Gin at position five of [D-Arg1,D-Trp7,9-Leu11]SP with D-Phe to form [D-Arg1,D-Phe5,D-Trp7,9,Gly11]SP resulted in a broad spectrum neuropeptide antagonist which was 5-fold more potent (44). We reasoned that further substitutions at this position may result in SP analogues with increased potency. We established that substitution of D-Phe at position 5 with D-Tyr did not significantly affect antagonistic activity against bombesin (data not shown). In this report, we describe the results of inhibitory monographs on DNA synthesis induced by various agents (see below). Cells from parallel cultures were incubated with anti-mouse IgG (1:1000) for anti-Tyr(P) mAbs or 1:125-labeled protein A (1:1000) for the MAP kinase antiserum.

**Measurement of Intracellular Calcium**—[Ca2+]i was measured with the fluorescent Ca2+ indicator fura-2/AME using a modification of the procedure previously described (52). Quiescent cells in 90-mm dishes were washed twice in DMEM and then incubated at 37 °C for 10 min in 5 ml of DMEM with 1 μM fura-2 tetraacetoxymethyl ester. The dishes were washed three times in phosphate-buffered saline at 37 °C, and the cells were then suspended in 2 ml of electrolyte solution containing 120 mM NaCl, 5 mM KCl, 1.8 mM CaCl2, 0.9 mM MgCl2, 25 mM glucose, 16 mM Hepes, 6 mM Tris, and an amino acid mixture equivalent to DMEM (pH 7.2) by gentle scraping and transferred to a quartz cuvette. The suspension was stirred continuously and maintained at 37 °C. Various factors were added as indicated in the figure legends. Fluorescence was monitored in a Perkin-Elmer LS-5 luminescence spectrophotometer with an excitation wavelength of 336 nm and emission wavelength of 510 nm. [Ca2+]i, was calculated using the formula [Ca2+]i = KF - Fmax(Fmax - F), where F is the fluorescence at the unknown [Ca2+]i, Fmax is the fluorescence after addition of 0.02% Triton X-100, and F is the fluorescence after the Ca2+ in the solution is chelated with 10 mM EGTA. Various inhibitors were added at a concentration of 290 mM, [D-Arg1,D-Phe5,D-Trp7,9,Val11]SP was kindly provided by Peptech, NSW, Australia. All other materials were of the highest grade available.

**Substance P Analogue Neuropeptide Inhibitors**

Bombesin-induced signal transduction pathways. This proposal was based on the fact that high concentrations of bombesin reversed the inhibition of MAP kinase but not PIP2-PLC-β activation caused by N2P2-PLC-β activation caused by [D-Arg1,D-Phe5,D-Trp7,9,Leu11]SP in Swiss 3T3 cells (49). In contrast, we have recently demonstrated that high concentrations of either bombesin or vasoressin reverse the inhibitory effect of this SP analogue on inositol phosphate production in Swiss 3T3 cells (50). These discrepant results prompted us to examine the inhibitory effect of several novel SP analogues on the multiple signal transduction pathways induced by bombesin and vasoressin in Swiss 3T3 cells.
We initially determined whether [D-Arg¹,D-Phe⁵,D-Trp⁷,⁹,Leu¹¹]SP had the same pattern of selectivity as [D-Arg¹,D-Phe⁵,D-Trp⁷,⁹,Leu¹¹]SP on DNA synthesis induced by various growth factors. As shown in Fig. 1, both SP analogues markedly inhibited DNA synthesis induced by bombesin, GRP, vasopressin, and bradykinin but did not affect mitogenesis stimulated by EGF forskolin and phorbol 12,13-dibutyrate. Interestingly, [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP was more effective than [D-Arg¹,D-Phe⁵,D-Trp⁷,⁹,Leu¹¹]SP at inhibiting DNA synthesis induced by bombesin, GRP, or bradykinin.

To compare the ability of [D-Arg¹,D-Phe⁵,D-Trp⁷,⁹,Leu¹¹]SP and [D-Arg¹,D-Trp⁷,⁹,Leu¹¹]SP to inhibit bombesin-stimulated DNA synthesis, confluent and quiescent Swiss 3T3 cells were incubated with increasing concentrations of bombesin in the absence or presence of these antagonists at 10 μM. The experiments were performed in the presence of insulin to ensure maximal induction of DNA synthesis by bombesin. Fig. 2 demonstrates that [D-Arg¹,D-Trp⁷,⁹,Leu¹¹]SP caused a 6-fold further rightward shift in the bombesin dose response curve than the same concentration of [D-Arg¹,D-Phe⁵,D-Trp⁷,⁹,Leu¹¹]SP. The ability of either [D-Arg¹,D-Phe⁵,D-Trp⁷,⁹,Leu¹¹]SP or [D-Arg¹,D-Phe⁵,D-Trp⁷,⁹,Leu¹¹]SP to inhibit [³H]thymidine incorporation was reversed by high concentrations of bombesin. Fig. 2 (inset) shows that [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP inhibited bombesin-stimulated DNA synthesis in a concentration-dependent manner with an IC₅₀ of 3 μM. Thus, [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP is more potent than [D-Arg¹,D-Phe⁵,D-Trp⁷,⁹,Leu¹¹]SP and the inhibitory effect of either SP analogue can be overcome by increasing concentrations of bombesin.

[D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP Reversibly Inhibits Bombesin-induced Ca²⁺ Mobilization and 80K/MARCKS phosphorylation—To examine the mechanism of action of [D-Arg¹,D-Phe⁵,D-Trp⁷,⁹,Leu¹¹]SP, we tested the inhibitory effect of this new, more potent, SP analogue on bombesin-induced signal transduction pathways. As shown in Fig. 3A (upper) 15 μM [D-Arg¹,D-Trp⁷,⁹,Leu¹¹]SP induced a 51-fold rightward shift in the dose response curve of bombesin-induced Ca²⁺ mobilization, which is induced via a Gα-mediated, PIP₂-PLC-β-dependent pathway. Importantly, high concentrations of bombesin could completely reverse the inhibitory effect of [D-Arg¹,D-Trp⁷,⁹,Leu¹¹]SP. In other experiments we found that the antagonistic effect of [D-Arg¹,D-Phe⁵,D-Trp⁷,⁹,Leu¹¹]SP on bombesin-stimulated Ca²⁺ mobilization could also be reversed in the presence of high ligand concentrations (data not shown). Furthermore, this reversible antagonism was not dependent on preincubation time as identical results were obtained when the SP analogue was added 5 min before, immediately before, or together with bombesin (data not shown). Fig. 3A (upper, inset) demonstrates that increasing concentrations of [D-Arg¹,D-Trp⁷,⁹,Leu¹¹]SP prevented Ca²⁺ mobilization induced by bombesin in a dose-dependent manner.

Another consequence of PIP₂-PLC-β activation is the generation of diacyl glycerol, which induces PKC-mediated 80K/MARCKS phosphorylation. Bombesin-induced 80K/MARCKS phosphorylation was markedly inhibited by 15 μM [D-Arg¹,D-Trp⁷,⁹,Leu¹¹]SP (Fig. 3A. lower). However, increasing the concentration of bombesin could completely reverse the inhibitory effect of [D-Arg¹,D-Trp⁷,⁹,Leu¹¹]SP. Thus the ability of [D-Arg¹,D-Trp⁷,⁹,Leu¹¹]SP to inhibit bombesin-induced Ca²⁺ mobilization or 80K/MARCKS phosphorylation can be over-
come in a competitive fashion by increasing concentrations of neuropeptide.

Effect of [D-Arg\textsubscript{1},D-Trp\textsubscript{5,7,9},Leu\textsubscript{11}]SP on Bombesin-induced MAP Kinase Activation—Next, we examined the effect of [D-Arg\textsubscript{1},D-Trp\textsubscript{5,7,9},Leu\textsubscript{11}]SP on MAP kinase activation stimulated by bombesin. Cultures of Swiss 3T3 cells incubated with increasing concentrations of bombesin for 5 min in the absence or presence of 15 \textmu M [D-Arg\textsubscript{1},D-Trp\textsubscript{5,7,9},Leu\textsubscript{11}]SP prior to lysis. The lysates were analyzed by immune complex assays of MAP kinase activity using myelin basic protein peptide as a substrate. Fig. 3B (upper) shows that 15 \textmu M [D-Arg\textsubscript{1},D-Trp\textsubscript{5,7,9},Leu\textsubscript{11}]SP induced a marked rightward shift in the dose-response curve for bombesin-induced MAP kinase activation. The increase in the EC\textsubscript{50} of bombesin-induced MAP kinase (40-fold) was similar to that of Ca\textsuperscript{2+} mobilization (51-fold) in the presence of [D-Arg\textsubscript{1},D-Trp\textsubscript{5,7,9},Leu\textsubscript{11}]SP (Fig. 3, upper).

In particular, the inability of [D-Arg\textsubscript{1},D-Trp\textsubscript{5,7,9},Leu\textsubscript{11}]SP to block MAP kinase activation was, just like Ca\textsuperscript{2+} mobilization, reversed at high concentrations of bombesin.

Fig. 3B (upper, inset) demonstrates that increasing concentrations of [D-Arg\textsubscript{1},D-Trp\textsubscript{5,7,9},Leu\textsubscript{11}]SP prevented MAP kinase activation induced by bombesin in a dose-dependent manner. [D-Arg\textsubscript{1},D-Trp\textsubscript{5,7,9},Leu\textsubscript{11}]SP did not alter the time course of bombesin-induced MAP kinase activation over a 60-min period. Maximum stimulation was achieved after 5 min of exposure to bombesin either in the absence or presence of the SP analogue.

data not shown.

To further substantiate the results obtained with the immune complex MAP kinase assay, lysates of Swiss 3T3 cells stimulated with bombesin in the absence or presence of [D-Arg\textsubscript{1},D-Trp\textsubscript{5,7,9},Leu\textsubscript{11}]SP were subjected to SDS-PAGE followed by Western blotting with anti-p42\textsuperscript{MAPK} antibody. The results shown are representative of three independent experiments.
100 nM bombesin. Furthermore, [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP also reversibly inhibited bombesin-stimulated tyrosine phosphorylation of p125FAK in Swiss 3T3 cells (Fig. 4, lower).

[D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP reversibly inhibits vasopressin-induced Ca²⁺ mobilization, 80K/MARCKS phosphorylation, and MAP kinase activation—The preceding data indicate that bombesin-induced signal transduction pathways are coordinate and reversibly inhibited by [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP. In order to substantiate these findings, we examined the effect of this antagonist on signal transduction pathways stimulated by occupancy of the distinct V₁ vasopressin receptor, which is also expressed by Swiss 3T3 cells (2). We initially established that [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP potently inhibited vasopressin-induced DNA synthesis in a reversible fashion in Swiss 3T3 cells (Fig. 1 and data not shown).

Fig. 5 shows that 1 µM [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP induced a marked rightward shift in the dose-response curves of vasopressin-induced Ca²⁺ mobilization (Fig. 5A, upper) and MAP kinase activation (Fig. 5B, upper). The increase in EC₅₀ was similar for the two responses measuring 17- and 15-fold, respectively. The inhibitory effect of [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP on both vasopressin-induced Ca²⁺ mobilization and MAP kinase activation was reversed by high concentrations of this neuropeptide. The insets show that [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP inhibited vasopressin-induced Ca²⁺ mobilization and MAP kinase activation in a dose-dependent fashion. The effect of [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP on vasopressin-stimulated MAP kinase activation was verified using the band shift assay (Fig. 5B, lower). In addition, [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP reversibly inhibited vasopressin-induced 80K/MARCKS phosphorylation (Fig. 5A, lower). Finally, we found that [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP could reversibly block vasopressin-induced tyrosine phosphorylation of multiple substrates, including p125FAK (data not shown). These results demonstrate that [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP, as previously seen with bombesin, coordinate inhibits vasopressin-induced mitogenesis, PIP₂-PLC-β stimulation, MAP kinase activation, and tyrosine phosphorylation.

[D-Arg¹,D-Phe⁵,D-Trp⁷,⁹]SP¹⁰ Reversibly Inhibits Vasopressin-but Not Bombesin-induced DNA Synthesis, Ca²⁺ Mobilization, and MAP Kinase Activation—Recently, it has been shown that SP analogues are metabolized predominantly by oxidation of the amino acid at the COOH terminus (56). To test the effect of additional SP analogues on neuropeptide-stimulated mitogenesis and signal transduction, we used synthetic peptide analogues that had substitutions or deletions of the terminal amino acid of [D-Arg¹,D-Phe⁵,D-Trp⁷,⁹,Leu¹¹]SP (shown in Fig. 6). Replacement of the terminal Leu of [D-Arg¹,D-Phe⁵,D-Trp⁷,⁹,Leu¹¹]SP with Val to produce [D-Arg¹,D-Phe⁵,D-Trp⁷,⁹,Val¹¹]SP did not change the inhibitory activity of the SP analogue against either bombesin- or vasopressin-induced mitogenesis (Fig. 6), Ca²⁺ mobilization, and MAP kinase activation (Fig. 7). In addition, the inhibitory effect of both SP analogues could be reversed by high concentrations of either neuropeptide (data not shown). In contrast, substitution of the terminal Leu with Gly resulted in a SP analogue with almost no inhibitory effect on mitogenesis, Ca²⁺ mobilization, and MAP kinase activation stimulated by either neuropeptide (Figs. 6 and 7).

Deletion of the terminal Leu to form [D-Arg¹,D-Phe⁵,D-Trp⁷,⁹]SP¹⁰ produced an antagonist which potently inhibited vasopressin-induced mitogenesis (Fig. 6), Ca²⁺ mobilization, and MAP kinase activation (Fig. 7). Surprisingly, [D-Arg¹,D-Phe⁵,D-Trp⁷,⁹,Leu¹¹]SP did not significantly block mitogenesis (Fig. 6) and only weakly inhibited Ca²⁺ mobilization and MAP kinase activation (Fig. 7) induced by bombesin, even at concentrations where these responses were completely inhibited by either [D-Arg¹,D-Phe⁵,D-Trp⁷,⁹,Leu¹¹]SP or [D-Arg¹,D-Phe⁵,D-Trp⁷,⁹,Val¹¹]SP. These results indicate that deletion of Leu¹¹ from [D-Arg¹,D-Phe⁵,D-Trp⁷,⁹,Leu¹¹]SP markedly reduces the broad spectrum activity of this antagonist since the SP analogue [D-Arg¹,D-Phe⁵,D-Trp⁷,⁹,Leu¹¹]SP¹⁰ coordinate inhibits vasopressin- but not bombesin-induced signal transduction and mitogenesis.

DISCUSSION

[D-Arg¹,D-Phe⁵,D-Trp⁷,⁹,Leu¹¹]SP has been previously shown to block the action of multiple neuropeptides including bombesin and vasopressin in Swiss 3T3 cells (4, 40–44) and to inhibit small cell lung cancer cell growth in vitro and as xenografts in vivo (46–48). Here, we demonstrate that substitution of D-Phe at position 5 with D-Trp to form [D-Arg¹,D-Trp⁷,⁹,Leu¹¹]SP produced a SP analogue with a further increase in potency against neuropeptide-induced mitogenesis. Importantly, this new SP analogue, like previously identified SP antagonists, inhibited DNA synthesis induced by bombesin, vasopressin, and bradykinin, but did not interfere with the mitogenic response induced by other growth factors or pharmacological agents.

Bombesin induces a rapid PLC-β-mediated hydrolysis of PIP₂ to produce the second messengers inositol 1,4,5-trisphosphate which promotes mobilization of Ca²⁺ from intracellular stores and diacyl glycerol which activates PKC (17–22). Bombesin also causes a striking activation of p42/p44 MAPK (27, 28). It is well established that these highly conserved kinases are activated by a wide range of stimuli through p21ras and PKC signaling pathways. While bombesin neither induces significant p21ras loading with GTP nor p74MAPK activation (49, 57) this neuropeptide promotes PKC-dependent activation of p42/p44MAPK in Swiss 3T3 cells (27, 57).

The results presented here show that [D-Arg¹,D-Trp⁷,⁹,Leu¹¹]SP reversibly inhibited Ca²⁺ mobilization and PKC-mediated 80K/MARCKS phosphorylation and by inference PIP₂-PLC-β activation induced by bombesin. This is in agreement with our previous findings demonstrating that [D-Arg¹,D-Phe⁵,D-Trp⁷,⁹,Leu¹¹]SP competitively inhibits inositol phosphate production in Swiss 3T3 cells (50). Furthermore, [D-Arg¹,D-Trp⁷,⁹,Leu¹¹]SP reversibly inhibited MAP kinase...
activation by bombesin, in accord with the model that places PIP_2-PLC-β, PKC, and p42MAPK in a common signal transduction pathway in bombesin-treated Swiss 3T3 cells.

A recent report has suggested that [d-Arg^1,d-Trp^5,7,9,Leu^11]SP can selectively inhibit the activation of PIP_2-PLC-β but not MAP kinase at high bombesin concentrations in Swiss 3T3 cells (49). It was proposed that disruption of the coordinate regulation of bombesin-induced signaling pathways contributes to the growth inhibitory properties of [d-Arg^1,d-Trp^5,7,9,Leu^11]SP (49). This implies that the growth-inhibitory effects of the SP analogues should not be reversed by high concentrations of agonist. However, our results demonstrate that 1) the inhibition of DNA synthesis by [d-Arg^1,d-Trp^5,7,9,Leu^11]SP was reversed by increasing concentrations of bombesin, 2) although the inhibition curves for [d-Arg^1,d-Trp^5,7,9,Leu^11]SP on bombesin-induced Ca^{2+} mobilization and MAP kinase activation were slightly different, the dose-response curves for bombesin-induced Ca^{2+} mobilization and MAP kinase activation were similarly displaced by the SP analogue, and 3) importantly, the inhibitory effect of the SP analogue on both Ca^{2+} mobilization and MAP kinase activation could be completely reversed at high bombesin concentrations. These results prompted us to perform additional experiments to test further the mechanism of action of [d-Arg^1,d-Trp^5,7,9,Leu^11]SP and other related peptides.

It is well established that bombesin induces a rapid increase in the tyrosine phosphorylation of multiple substrates including p125FAK (29–35), through a signal transduction pathway that is mediated by p21Rho (58, 59). This pathway is not dependent on either PKC activation or Ca^{2+} mobilization (32, 34, 60). Recently, we verified that the bombesin receptor transfected and stably expressed in Rat-1 cells mediates Ca^{2+} mobilization, PKC activation as well as tyrosine phosphorylation of multiple substrates including p125FAK (61). In the present study we demonstrate that [d-Arg^1,d-Trp^5,7,9,Leu^11]SP reversibly inhibited bombesin-induced tyrosine phosphorylation of multiple substrates including p125FAK in Swiss 3T3 cells. These data support the proposition that this SP analogue co-ordinately inhibits the activation of the signal transduction pathways emanating from the bombesin receptor.

Vasopressin binds to a distinct G_α coupled receptor that also induces PIP_2-PLC-mediated Ca^{2+} mobilization, 80K/MARCKS phosphorylation, MAP kinase activation, and tyrosine phosphorylation of multiple substrates including p125FAK in Swiss 3T3 cells (reviewed in Ref. 2). In order to substantiate our findings with bombesin, we also studied the effect of [d-Arg^1,d-Trp^5,7,9,Leu^11]SP on vasopressin-stimulated signaling events. As previously seen with bombesin, we found that this SP ana-
logue competitively inhibited vasopressin-induced Ca\(^{2+}\) mobilization, 80K/MARCKS phosphorylation, MAP kinase activation, tyrosine phosphorylation, and reincarnation of DNA synthesis. In particular, the dose responses for vasopressin-induced Ca\(^{2+}\) mobilization and MAP kinase activation were similarly displaced by [D-Arg\(^1\), D-Trp\(^{5,7,9}\), Leu\(^{11}\)]SP.

The coordinate inhibition of neuropeptide stimulated signal transduction pathways could be a feature specific to the new SP analogue, [D-Arg\(^1\), D-Trp\(^{5,7,9}\), Leu\(^{11}\)]SP, rather than a common property of all SP analogue antagonists. We verified that [D-Arg\(^1\), D-Phe\(^{3}\), D-Trp\(^{7,9}\), Leu\(^{11}\)]SP could also competitively block bombesin- and vasopressin-induced mitogenesis, Ca\(^{2+}\) mobilization, and MAP kinase activation. Furthermore, we examined the effect of additional SP analogues generated by substitutions or deletion of the terminal amino acid of [D-Arg\(^1\), D-Phe\(^{3}\), D-Trp\(^{7,9}\), Leu\(^{11}\)]SP. The conservative substitution of Leu\(^{11}\) with Val yielded a peptide that behaved identically to [D-Arg\(^1\), D-Phe\(^{3}\), D-Trp\(^{7,9}\), Leu\(^{11}\)]SP and [D-Arg\(^1\), D-Trp\(^{7,9}\), Leu\(^{11}\)]SP. Thus, three different SP analogues inhibit neuropeptide-induced mitogenesis, Ca\(^{2+}\) mobilization, and MAP kinase activation in a reversible and coordinate fashion.

A model that accounts for the coordinate inhibition of bombesin or vasopressin stimulated signal transduction by [D-Arg\(^1\), D-Phe\(^{3}\), D-Trp\(^{7,9}\), Leu\(^{11}\)]SP and related peptides is that these SP analogues interfere with agonist binding to their receptors. In fact, we have previously shown that SP analogues competitively inhibit ligand binding (50), but these findings could not rule out an indirect mechanism mediated by uncoupling of a G protein from the receptor. Surprisingly, deletion of the terminal Leu of [D-Arg\(^1\), D-Phe\(^{3}\), D-Trp\(^{7,9}\), Leu\(^{11}\)]SP to form [D-Arg\(^1\), D-Phe\(^{3}\), D-Trp\(^{7}\), Leu\(^{11}\)]SP has provided novel mechanistic insight into this problem. We found that [D-Arg\(^1\), D-Phe\(^{3}\), D-Trp\(^{7}\), Leu\(^{11}\)]SP to [D-Arg\(^1\), D-Phe\(^{3}\), D-Trp\(^{7}\), Val\(^{11}\)]SP. Therefore, the SP analogue [D-Arg\(^1\), D-Phe\(^{3}\), D-Trp\(^{7,9}\)]SP coordinately inhibits vasopressin but not bombesin-induced signal transduction. This differential modulation strongly suggests that the truncated SP analogue acts as a potent vasopressin (but not bombesin) receptor antagonist. Our results imply that these inhibitory molecules block neuropeptide-mediated signal transduction at the receptor level.

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