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[D-Arg^1,D-Trp^{5,7,9},Leu^{11}]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-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP like [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]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-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP reversibly inhibited bombesin-induced DNA synthesis, causing a 6-fold greater rightward shift in the bombesin dose response than [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]SP at identical concentrations (10 μM). We found that the new, more potent, SP analogue coordinately and reversibly inhibited bombesin-induced Ca^{2+} mobilization and protein kinase C (PKC) and mitogen-activated protein (MAP) kinase activation. The dose-response curves for bombesin-induced Ca^{2+} mobilization and MAP kinase activation were similarly displaced (51- and 40-fold, respectively) by [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP. In addition, [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP reversibly inhibited bombesin-induced tyrosine phosphorylation of M_r 110,000–130,000 and 70,000–80,000 bands as well as p125 focal adhesion kinase. [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP also reversibly and coordinately inhibited vasopressin-induced Ca^{2+} mobilization, PKC stimulation, MAP kinase activation, tyrosine phosphorylation, and DNA synthesis in Swiss 3T3 cells. Surprisingly, deletion of the terminal Leu of [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]SP to yield [D-Arg^1,D-Phe^5,D-Trp^{7,9}]SP$^{1-10}$ resulted in a selective loss of inhibitory activity of this analogue against bombesin- but not vasopressin-stimulated DNA synthesis, Ca^{2+} 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 $G_{\alpha q}$ subfamily (8–10) which stimulate the PIP_2-PLC-β isoforms of PLC (11–16). This results in rapid hydrolysis of polyphosphoinositides, with the consequent mobilization of Ca^{2+} 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-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]SP and [Arg^6,D-Trp^{7,9},MePhe^8]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 G_s, or platelet-derived growth factor, which signals through receptors with intrinsic tyrosine kinase activity (45). More recently, [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]SP and [Arg^6,D-Trp^{7,9},MePhe^8]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-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]SP selectively uncouples PIP_2-PLC-β 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

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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/AME, Fura 2-tetraacetoxymethyl ester; G protein, guanine nucleotide-binding regulatory protein; G_q, designation of class of heterotrimeric proteins; G_s, a heterotrimeric G protein that mediates stimulation of adenylate cyclase; mAb, monoclonal antibody; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; 80K/MARCKS, 80K/myristoylated alanine-rich protein kinase C substrate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC-β, specific phospholipase C-β isoform; PKC, protein kinase C; PAGE, polyacrylamide gel electrophoresis; SP, substance P; Hepps, 4-(2-hydoxyethyl)-1-piperazineethanesulfonic acid.

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 PIP_2-PLC-β activation caused by [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]SP in Swiss 3T3 cells (49). In contrast, we have recently demonstrated that high concentrations of either bombesin or vasopressin 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 vasopressin in Swiss 3T3 cells.

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% CO_2 and 90% air at 37 °C. For experimental purposes, cells were plated in 33- or 90-mm Nunc Petri dishes at 10^5 cells or 6×10^5 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 [^{3}H]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 [^{3}H]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—$[Ca^{2+}]_i$ was measured with the fluorescent Ca^{2+} 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 $CaCl_2$, 0.9 mM $MgCl_2$, 25 mM glucose, 16 mM Hepps, 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. $[Ca^{2+}]_i$ was calculated using the formula $[Ca^{2+}]_i$ nM $= K(F - F_{min})/(F_{max} - F)$, where F is the fluorescence at the unknown $[Ca^{2+}]_i$, F_{max} is the fluorescence after addition of 0.02% Triton X-100, and F_{min} is the fluorescence after the Ca^{2+} in the solution is chelated with 10 mM EGTA. The value of K was 220 nM for fura-2/AME (52).

^{32}P-Labeling of Cells and Analysis of 80K/MARCKS Phosphorylation—Quiescent and confluent cultures in 33-mm dishes were washed twice in phosphate-free DMEM and incubated at 37 °C with this medium containing 50 μCi/ml of carrier-free [^{32}P]P_i. After 18 h, various factors were added for the indicated times. The cells were then lysed in 500 μl/dish of a solution containing 10 mM Tris/HCl, pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 100 μM Na_3VO_4, 50 mM phenylmethylsulfonyl fluoride, and 0.5% Triton X-100 (lysis buffer) supplemented with 3.5 μg/ml aprotinin and 1 μg/ml leupeptin and the lysates were clarified by centrifugation for at 15,000 $\times$ g for 20 min at 4 °C. The supernatants were then immunoprecipitated with specific anti-80K/MARCKS antibody (53) and the immunoprecipitates were further analyzed by SDS-PAGE prior to autoradiography.

Immunoprecipitation of Tyrosine Phosphorylated Proteins—Quiescent and confluent cultures of cells in 33-mm dishes were washed twice with DMEM, and then treated with factors as indicated in the figure legends prior to lysis at 4 °C in 1 ml of lysis buffer. Lysates were centrifuged at 15,000 $\times$ g for 20 min, and the supernatants were incubated for 4 h at 4 °C with anti-mouse IgG agarose-linked mAbs directed against phosphotyrosine (Py72). The immunoprecipitates were washed three times with lysis buffer and further analyzed by SDS-PAGE and Western blotting (see below). Cells from parallel cultures treated in an identical fashion were suspended by trypsinization and counted using a Coulter counter to ensure equal numbers of cells per condition.

p42^{MAPK} Shift Assays and Western Blotting—Quiescent cultures of Swiss 3T3 cells were treated with factors as indicated and cells were lysed in 2 $\times$ SDS-PAGE-sample buffer (200 mM Tris/HCl, 6% SDS, 2 mM EDTA, 4% 2-mercaptoethanol, 10% glycerol, pH 6.8) and resolved by SDS-PAGE. After SDS-PAGE, proteins were transferred to Immobilon membranes. Membranes were blocked using 5% nonfat dried milk in phosphate-buffered saline, pH 7.2, and incubated for 1 h at 22 °C with either the anti-Tyr(P) mAbs (Py20 and 4G10, 1 μg/ml antibody), or the anti-p125^{FAK} mAb (1:1000), or a polyclonal p42^{MAPK} antiserum raised against a synthetic peptide to the COOH-terminal domain of p42^{MAPK} (1:1000) in phosphate-buffered saline containing 3% nonfat dried milk. Immunoreactive bands were visualized by autoradiography using either ^{125}I-labeled sheep anti-mouse IgG (1:1000) for anti-Tyr(P) mAbs or ^{125}I-labeled protein A (1:1000) for the MAP kinase antiserum.

Immune Complex Assay of p42^{MAPK} Activity—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 $\times$ g for 20 min at 4 °C, and the supernatants were immunoprecipitated using the polyclonal anti-p42^{MAPK} antibody together with protein A-agarose beads (40 μl, 1:1 slurry) for 2 h. Immune complexes were collected by centrifugation and washed once in lysis buffer and three times in kinase buffer (15 mM Tris-HCl, 15 mM $MgCl_2$). 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 μM ATP, 100 μCi/ml [γ-^{32}P]ATP, and 200 μM microcystin LR. 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 vasopressin alone in the absence of inhibitors. The specific activity of [γ-^{32}P]ATP used was 900-1200 cpm/pmol.

SDS-PAGE—Slab gel electrophoresis was performed essentially according to the method of Laemmli (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 ml) 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/MARCKS experiments were fixed in 25% methanol, 10% acetic acid (v/v) prior to drying under vacuum for 2 h at 80 °C. Radioactivity was detected at −70 °C using Fuji x-ray film with exposure times of 12–72 h.

Materials—Bombesin, vasopressin, EGF, and IgG-agarose were obtained from Sigma. Protein A-agarose was from Boehringer Mannheim. Anti-Tyr(P) mAb clone Py72 was obtained from the hybridoma development unit, Imperial Cancer Research fund, London, UK. PY20 anti-Tyr(P) mAb was from ICN. 4G10 anti-Tyr(P) mAb was from Upstate Biotechnology Inc., Lake Pladid, NY. The anti-p125^{FAK} mAb for Western blotting was obtained from AFFINITI Research Products Ltd., Nottingham, UK. The polyclonal anti-p42^{MAPK} (anti-ERK-2) antibody raised against a COOH-terminal peptide (EETARFQPGYRS) was a generous gift from Dr J. Van Lint (Katholieke Universiteit Leuven, Belgium). ^{125}I-Labeled sheep anti-mouse immunoglobulin G (15 mCi/mg), ^{125}I-labeled protein A (15 mCi/mg), carrier-free [^{32}P]P_i (370 MBq/ml), [γ-^{32}P]ATP (370 MBq/ml), and [^{3}H]thymidine were from Amersham (UK). Fura 2/AME and forskolin were from Calbiochem. Methylisobutylxanthine was purchased from Aldrich. [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]SP was from Bachem California, Torrance, CA. [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP, [D-Arg^1,D-Phe^5,D-Trp^{7,9},Val^{11}]SP, [D-Arg^1,D-Phe^5,D-Trp^{7,9},Gly^{11}]SP, and [D-Arg^1,D-Phe^5,D-Trp^{7,9}]SP$^{1-10}$ were kindly provided by Peptech, NSW, Australia. All other materials were of the highest grade available.

RESULTS

Comparison of the Inhibitory Effect of [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP with [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]SP on DNA Synthesis Induced by Various Agents—We have previously shown that substitution of Gln at position five of [D-Arg^1,D-Trp^{7,9},Leu^{11}]SP with D-Phe to form [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]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 contrast, when this residue was replaced by D-Trp to form [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP an increase in inhibitory activity was obtained (see below). In view of these results, [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP was selected for further investigation.

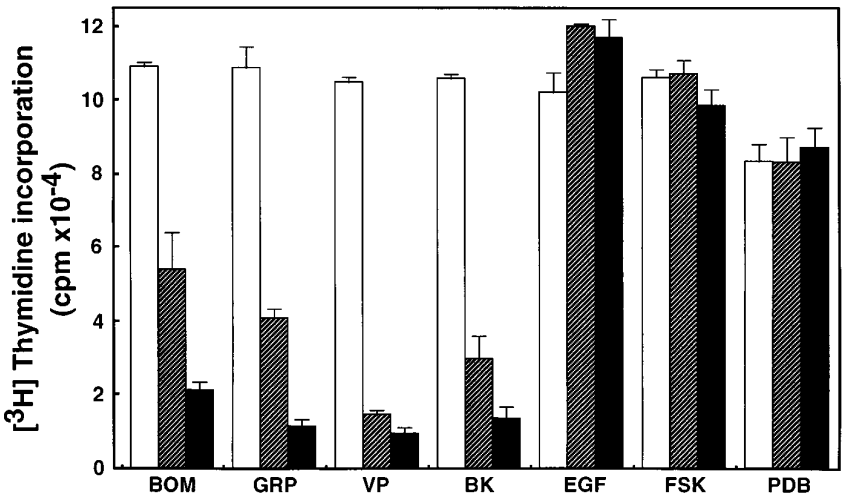

FIG. 1. **Effect of [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]SP and [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP on the stimulation of DNA synthesis induced by various mitogens in Swiss 3T3 cells.** Confluent and quiescent cultures of Swiss 3T3 cells in 33-mm dishes were stimulated with various factors in the presence of 0.5 μg/ml insulin in the absence or presence of either [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]SP (*hatched bars*) or [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP (*closed bars*) both at 20 μM. The factors were: 1 nM bombesin (*BOM*), 1 nM GRP, 1 nM vasopressin (*VP*), 10 nM bradykinin (*BK*), 5 ng/ml EGF, 10 μM forskolin with 50 μM isobutylmethylxanthine (*FSK*), and 200 nM phorbol 12,13-dibutyrate. [^{3}H]Thymidine incorporation was measured at 40 h by incorporation into acid-insoluble material. □, control. Each bar represents the mean of three independent experiments done in duplicate ± S.E.

We initially determined whether [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP had the same pattern of selectivity as [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]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-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP was more effective than [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]SP at inhibiting DNA synthesis induced by bombesin, GRP, or bradykinin.

To compare the ability of [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]SP and [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]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-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP caused a 6-fold further rightward shift in the bombesin dose response curve than the same concentration of [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]SP. The ability of either [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP or [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]SP to inhibit [^{3}H]thymidine incorporation was reversed by high concentrations of bombesin. Fig. 2 (*inset*) shows that [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP inhibited bombesin-stimulated DNA synthesis in a concentration-dependent manner with an IC_{50} of 3 μM. Thus, [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP is more potent than [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]SP, and the inhibitory effect of either SP analogue can be overcome by increasing concentrations of bombesin.

[D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP Reversibly Inhibits Bombesin-induced Ca^{2+} Mobilization and 80K/MARCKS Phosphorylation—To examine the mechanism of action of [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]SP, we tested the inhibitory effect of this new, more potent, SP analogue on bombesin-induced signal transduction pathways. As shown in Fig. 3*A* (*upper*) 15 μM [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP induced a 51-fold rightward shift in the dose response curve of bombesin-induced Ca^{2+} mobilization, which is induced via a G_q-mediated, PIP_2-PLC-β-dependent pathway. Importantly, high concentrations of bombesin could completely reverse the inhibitory effect of [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP. In other experiments we found that the antagonistic effect of [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]SP on bombesin-stimulated Ca^{2+} 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. 3*A* (*upper*, *inset*) demonstrates that increasing concentrations of [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP prevented Ca^{2+} mobilization induced by bombesin in a dose-dependent manner.

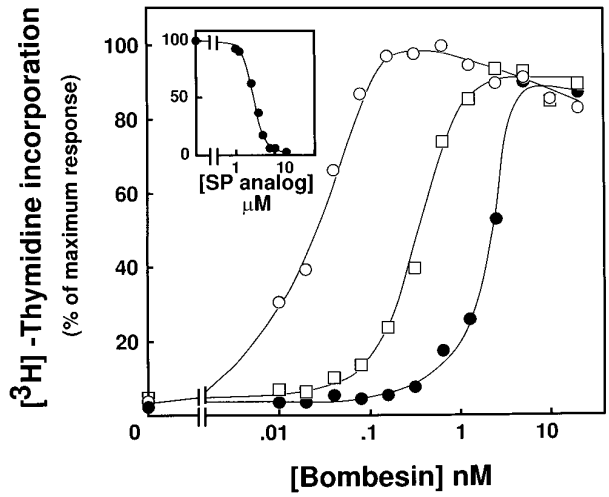

FIG. 2. **Dose-response curves for the stimulatory effect of bombesin on DNA synthesis in the absence or presence of [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP and [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]SP.** Cultures of Swiss 3T3 cells in 33-mm dishes were stimulated with increasing concentrations of bombesin in the absence (*open circles*) or presence of [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]SP (*open squares*) or [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP (*closed circles*) both at 10 μM. *Inset*, dose-response curve for the inhibitory effect of [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP on bombesin-induced DNA synthesis. Cultures of Swiss 3T3 cells in 33-mm dishes were stimulated with 0.5 nM bombesin in the absence or presence of increasing concentrations of [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP. All experiments were performed in the presence of 0.5 μg/ml insulin. [^{3}H]Thymidine incorporation was measured at 40 h by incorporation into acid-insoluble material. In all cases, a representative of three independent experiments is shown. Each point is the mean of duplicate samples expressed as the percentage of the maximum stimulation induced by bombesin in the absence of SP analogue. Maximum stimulation was 120,000 cpm/culture less a background value of 4500 cpm/culture in the absence of any added factors.

Another consequence of PIP_2-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-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP (Fig. 3*A*. *lower*). However, increasing the concentration of bombesin could completely reverse the inhibitory effect of [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP. Thus the ability of [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP to inhibit bombesin-induced Ca^{2+} mobilization or 80K/MARCKS phosphorylation can be over-

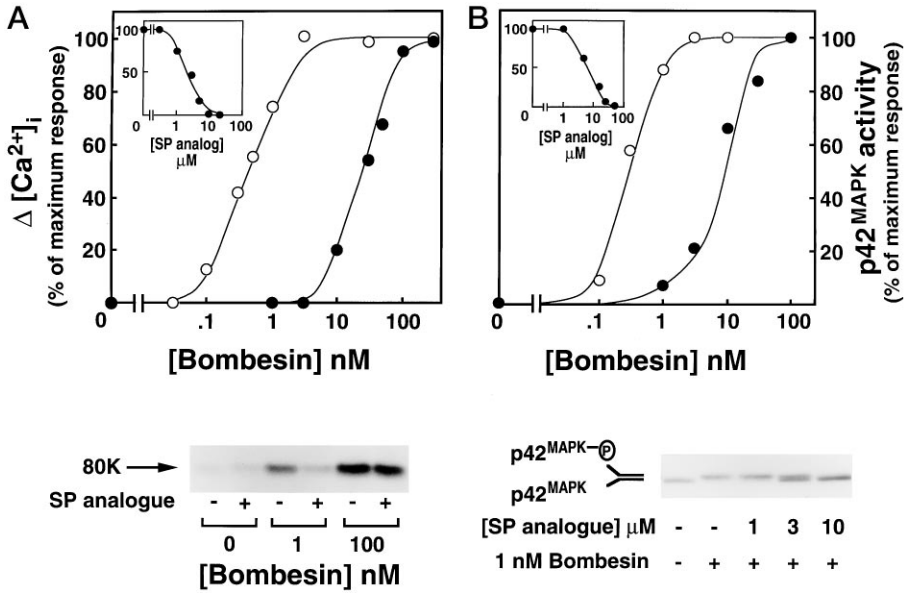

FIG. 3. *Panel A*, *upper*, effect of [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP on bombesin-induced Ca^{2+} mobilization. Confluent and quiescent Swiss 3T3 cells loaded with fura-2/AME were transferred to a quartz cuvette and then stimulated with increasing concentrations of bombesin in the absence (*open circles*) or presence (*closed circles*) of 15 μM [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP. *Inset*, Parallel cultures of Swiss 3T3 cells loaded with fura-2/AME were stimulated with 3 nM bombesin in the absence or presence of increasing concentrations of [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP. In all cases, intracellular Ca^{2+} was measured as described under "Experimental Procedures." Each point is representative of at least three independent experiments performed in duplicate and is expressed as a percentage of the maximum increase in intracellular Ca^{2+} induced by 3 nM bombesin ($\Delta[Ca^{2+}]_i$ 640 ± 27 nM with an unstimulated value of 140 ± 10 nM; n = 5 ± S.E.). *Lower*, effect of [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP on bombesin-induced 80K/MARCKS phosphorylation. Confluent and quiescent Swiss 3T3 cells in 33-mm dishes were incubated with 50 μCi of [^{32}P]P_i for 18 h and then stimulated for 10 min with either 1 or 100 nM bombesin in the absence or presence of 15 μM [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP. The cultures were then lysed and the lysates immunoprecipitated with specific anti-80K/MARCKS antibody prior to SDS-PAGE electrophoresis as described under "Experimental Procedures." The 80K/MARCKS band is *arrowed* and control unstimulated lanes with or without [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP are also shown. *Panel B*, *upper*, effect of [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP on bombesin-induced MAP kinase activation. Confluent and quiescent Swiss 3T3 cells in 90-mm dishes were stimulated for 5 min with increasing concentrations of bombesin in the absence (*open circles*) or presence (*closed circles*) of 15 μM [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP. *Inset*, in parallel experiments, cultures of Swiss 3T3 cells were stimulated for 5 min with 3 nM bombesin in the absence or presence of increasing concentrations of [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP. In all cases, the cultures were then lysed and the lysates immunoprecipitated with a polyclonal anti-p42^{MAPK} antibody prior to analysis of MAP kinase activity as described under "Experimental Procedures." A representative of three independent experiments is shown. Each point is the mean of duplicate samples expressed as the percentage of the maximum stimulation induced by bombesin in the absence of SP analogue. The maximum stimulation was 10 ± 0.8-fold (n = 5 ± S.E.) above unstimulated control levels. *Lower*, confluent and quiescent Swiss 3T3 cells in 33-mm dishes were stimulated for 5 min with 1 nM bombesin in the absence or presence of increasing concentrations of [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP. The cultures were then lysed in 2 × SDS-sample buffer, and the lysates were run on SDS-PAGE prior to Western blotting with anti-p42^{MAPK} antibody. The results shown are representative of three independent experiments.

come in a competitive fashion by increasing concentrations of neuropeptide.

Effect of [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP on Bombesin-induced MAP Kinase Activation—Next, we examined the effect of [D-Arg^1,D-Trp^{5,7,9},Leu^{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 μM [D-Arg^1,D-Trp^{5,7,9},Leu^{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. 3*B* (*upper*) shows that 15 μM [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP induced a marked rightward shift in the dose-response curve for bombesin-induced MAP kinase activation. The increase in the EC_{50} of bombesin-induced MAP kinase (40-fold) was similar to that of Ca^{2+} mobilization (51-fold) in the presence of [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP (Fig. 3, *upper*). In particular, the ability of [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP to block MAP kinase activation was, just like Ca^{2+} mobilization, reversed at high concentrations of bombesin.

Fig. 3*B* (*upper*, *inset*) demonstrates that increasing concentrations of [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP prevented MAP kinase activation induced by bombesin in a dose-dependent manner. [D-Arg^1,D-Trp^{5,7,9},Leu^{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^1,D-Trp^{5,7,9},Leu^{11}]SP were subjected to SDS-PAGE followed by Western blotting with anti-MAP kinase antibody (55). The activation of MAP kinase induced by bombesin was inhibited by increasing concentrations of the SP analogue as judged by the disappearance of slower migrating forms in the mobility shift assay (Fig. 3, *lower*).

[D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP Reversibly Inhibits Bombesin-induced Tyrosine Phosphorylation—Bombesin rapidly induces tyrosine phosphorylation of a number of substrates including bands migrating with an apparent M_r of 110,000–130,000 and M_r 70,000–80,000 in Swiss 3T3 cells (30, 31, 35). The focal adhesion associated tyrosine kinase p125^{FAK} has been identified as a component of the M_r 110,000–130,000 group of proteins (32–35). To test further the hypothesis that [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP can coordinately and reversibly inhibit bombesin-induced signaling pathways we also examined the effect of this SP analogue on bombesin-induced tyrosine phosphorylation. As shown in Fig. 4 (*upper*), 10 μM [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP inhibited tyrosine phosphorylation of both the M_r 110,000–130,000 and M_r 70,000–80,000 bands induced by 1 nM bombesin. The inhibitory effect of 10 μM [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP was completely reversed in the presence of

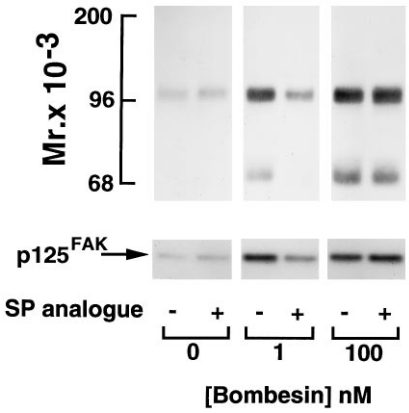

FIG. 4. **[D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP reversibly inhibits tyrosine phosphorylation of multiple substrates including p125^{FAK} induced by bombesin.** Confluent and quiescent Swiss 3T3 cells in 90-mm dishes were stimulated for 10 min with either 1 nM or 100 nM bombesin in the absence or presence of 10 μM [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP. The cells were then lysed and the lysates immunoprecipitated with the anti-Tyr(P) mAb Py72. The immunoprecipitates were further analyzed by SDS-PAGE and Western blotting with either anti-phosphotyrosine mAbs (*upper*) or an anti-p125^{FAK} mAb (*lower*). Representative autoradiographs of three independent experiments are shown.

100 nM bombesin. Furthermore, [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP also reversibly inhibited bombesin-stimulated tyrosine phosphorylation of p125^{FAK} in Swiss 3T3 cells (Fig. 4, *lower*).

[D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP Reversibly Inhibits Vasopressin-induced Ca^{2+} Mobilization, 80K/MARCKS Phosphorylation, and MAP Kinase Activation—The preceding data indicate that bombesin-induced signal transduction pathways are coordinately and reversibly inhibited by [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]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-Arg^1,D-Trp^{5,7,9},Leu^{11}]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-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP induced a marked rightward shift in the dose-response curves of vasopressin-induced Ca^{2+} mobilization (Fig. 5*A*, *upper*) and MAP kinase activation (Fig. 5*B*, *upper*). The increase in EC_{50} was similar for the two responses measuring 17- and 15-fold, respectively. The inhibitory effect of [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP on both vasopressin-induced Ca^{2+} mobilization and MAP kinase activation was reversed by high concentrations of this neuropeptide. The *insets* show that [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP inhibited vasopressin-induced Ca^{2+} mobilization and MAP kinase activation in a dose-dependent fashion. The effect of [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP on vasopressin-stimulated MAP kinase activation was verified using the band shift assay (Fig. 5*B*, *lower*). In addition, [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP reversibly inhibited vasopressin-induced 80K/MARCKS phosphorylation (Fig. 5*A*, *lower*). Finally, we found that [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP could reversibly block vasopressin-induced tyrosine phosphorylation of multiple substrates, including p125^{FAK} (data not shown). These results demonstrate that [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP, as previously seen with bombesin, coordinately inhibits vasopressin-induced mitogenesis, PIP_2-PLC-β stimulation, MAP kinase activation, and tyrosine phosphorylation.

[D-Arg^1,D-Phe^5,D-Trp^{7,9}]SP$^{1-10}$ Reversibly Inhibits Vasopressin- but Not Bombesin-induced DNA Synthesis, Ca^{2+} 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 which had substitutions or deletions of the terminal amino acid of [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]SP (shown in Fig. 6). Replacement of the terminal Leu of [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]SP with Val to produce [D-Arg^1,D-Phe^5,D-Trp^{7,9},Val^{11}]SP did not change the inhibitory activity of the SP analogue against either bombesin- or vasopressin-induced mitogenesis (Fig. 6), Ca^{2+} 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, Ca^{2+} mobilization, and MAP kinase activation stimulated by either neuropeptide (Figs. 6 and 7).

Deletion of the terminal Leu to form [D-Arg^1,D-Phe^5,D-Trp^{7,9}]SP$^{1-10}$ produced an antagonist which potently inhibited vasopressin-induced mitogenesis (Fig. 6), Ca^{2+} mobilization, and MAP kinase activation (Fig. 7). Surprisingly, [D-Arg^1,D-Phe^5,D-Trp^{7,9}]SP$^{1-10}$ did not significantly block mitogenesis (Fig. 6) and only weakly inhibited Ca^{2+} mobilization and MAP kinase activation (Fig. 7) induced by bombesin, even at concentrations where these responses were completely inhibited by either [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]SP or [D-Arg^1,D-Phe^5,D-Trp^{7,9},Val^{11}]SP. These results indicate that deletion of Leu^{11} from [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]SP markedly reduces the broad spectrum activity of this antagonist since the SP analogue [D-Arg^1,D-Phe^5,D-Trp^{7,9}]SP$^{1-10}$ coordinately inhibits vasopressin- but not bombesin-induced signal transduction and mitogenesis.

DISCUSSION

[D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]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-Arg^1,D-Trp^{5,7,9},Leu^{11}]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 PIP_2 to produce the second messengers inositol 1,4,5-trisphosphate which promotes mobilization of Ca^{2+} from intracellular stores and diacyl glycerol which activates PKC (17–22). Bombesin also causes a striking activation of p42^{MAPK} and p44^{MAPK} (27, 28, 55). It is well established that these highly conserved kinases are activated by a wide range of stimuli through p21^{ras} and PKC signaling pathways. While bombesin neither induces significant p21^{ras} loading with GTP nor p74^{raf} activation (49, 57) this neuropeptide promotes PKC-dependent activation of p42^{MAPK} in Swiss 3T3 cells (27, 57).

The results presented here show that [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP reversibly inhibited Ca^{2+} mobilization and PKC-mediated 80K/MARCKS phosphorylation and by inference PIP_2-PLC-β activation induced by bombesin. This is in agreement with our previous findings demonstrating that [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]SP competitively inhibits inositol phosphate production in Swiss 3T3 cells (50). Furthermore, [D-Arg^1,D-Trp^{5,7,9},Leu^{11}]SP reversibly inhibited MAP kinase

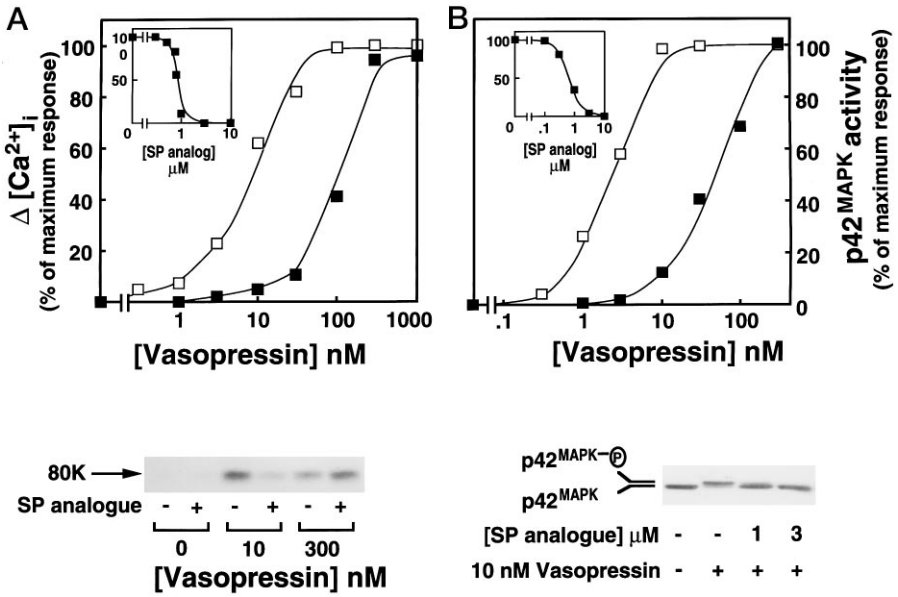

FIG. 5. *Panel A*, *upper*, dose-response curves for the inhibitory effect of vasopressin on Ca^{2+} mobilization in the absence or presence of [D-Arg^1,D-$Trp^{5,7,9}$,Leu^{11}]SP. Confluent and quiescent Swiss 3T3 cells loaded with fura-2/AME were transferred to a quartz cuvette and then stimulated with increasing concentrations of vasopressin in the absence (*open symbols*) or presence (*closed symbols*) of 1 μM [D-Arg^1,D-$Trp^{5,7,9}$,Leu^{11}]SP. *Inset*, dose-response curve for the inhibitory effect of [D-Arg^1,D-$Trp^{5,7,9}$,Leu^{11}]SP on vasopressin-induced Ca^{2+} mobilization. Swiss 3T3 cells loaded with fura-2/AME were stimulated with 10 nM vasopressin in the absence or presence of increasing concentrations of [D-Arg^1,D-Trp^5,D-$Trp^{7,9}$,Leu^{11}]SP. In all cases, intracellular Ca^{2+} was measured as described under "Experimental Procedures." Each point is representative of three independent experiments performed in duplicate and is expressed as a percentage of the maximum increase in intracellular Ca^{2+} induced by 100 nM (10 nM for *inset*) vasopressin (Δ $[Ca^{2+}]_i$ 560 $\pm$ 35 nM with an unstimulated control level of 140 $\pm$ 26 nM; $n = 3 \pm$ S.E.). *Lower*), effect of [D-Arg^1,D-$Trp^{5,7,9}$,Leu^{11}]SP on vasopressin-induced 80K/MARCKS phosphorylation. Confluent and quiescent Swiss 3T3 cells in 33-mm dishes were incubated with 50 μCi of [^{32}P]P_i for 18 h and then stimulated with either 10 or 300 nM vasopressin in the absence or presence of 1 μM [D-Arg^1,D-$Trp^{5,7,9}$,Leu^{11}]SP. After 10 min of incubation, the cultures were lysed, and the lysates were immunoprecipitated with specific anti-80K/MARCKS antibody prior to SDS-PAGE electrophoresis as described under "Experimental Procedures." The 80K/MARCKS band is *arrowed* and control unstimulated lanes with or without [D-Arg^1,D-$Trp^{5,7,9}$,Leu^{11}]SP are also shown. *Panel B*, *upper*, effect of [D-Arg^1,D-$Trp^{5,7,9}$,Leu^{11}]SP on vasopressin-induced MAP kinase activation. Confluent and quiescent Swiss 3T3 cells in 90-mm dishes were stimulated for 5 min with increasing concentrations of vasopressin in the absence (*open symbols*) or presence (*closed symbols*) of 1 μM [D-Arg^1,D-$Trp^{5,7,9}$,Leu^{11}]SP. *Inset*, dose-response curve for the effects of [D-Arg^1,D-$Trp^{5,7,9}$,Leu^{11}]SP on vasopressin-induced MAP kinase activation. In parallel experiments cultures of Swiss 3T3 cells were stimulated for 5 min with 10 nM vasopressin in the absence or presence of increasing concentrations of [DArg^1,D-$Trp^{5,7,9}$,Leu^{11}]SP. Cell lysates were immunoprecipitated with a polyclonal anti-$p42^{MAPK}$ antibody prior to analysis of MAP kinase activity as described in Experimental Procedures. A representative of three independent experiments is shown. Each point is the mean of duplicate samples expressed as the percentage of the maximum stimulation induced by vasopressin in the absence of SP analogue. The maximum response was 6.8 $\pm$ 1.2 fold ($n = 3 \pm$ S.E.) above unstimulated control levels. *Lower*, effect of [D-Arg^1,D-$Trp^{5,7,9}$,Leu^{11}]SP on vasopressin-induced MAP kinase activation assessed by shift assay. Confluent and quiescent Swiss 3T3 cells in 33-mm dishes were stimulated for 5 min with 10 nM vasopressin in the absence or presence of increasing concentrations of [D-Arg^1,D-$Trp^{5,7,9}$,Leu^{11}]SP. The cultures were then lysed in 2 $\times$ SDS-sample buffer and the lysates run on SDS-PAGE prior to Western blotting with anti-$p42^{MAPK}$ antibody. The results shown are representative of three independent experiments.

activation by bombesin, in accord with the model that places PIP_2-PLC-β, PKC, and $p42^{MAPK}$ in a common signal transduction pathway in bombesin-treated Swiss 3T3 cells.

A recent report has suggested that [D-Arg^1,D-Phe^5,D-$Trp^{7,9}$,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-Phe^5,D-$Trp^{7,9}$,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 $p125^{FAK}$ (29–35), through a signal transduction pathway that is mediated by $p21^{Rho}$ (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 $p125^{FAK}$ (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 $p125^{FAK}$ in Swiss 3T3 cells. These data support the proposition that this SP analogue coordinately inhibits the activation of the signal transduction pathways emanating from the bombesin receptor.

Vasopressin binds to a distinct G_q-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 $p125^{FAK}$ 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-

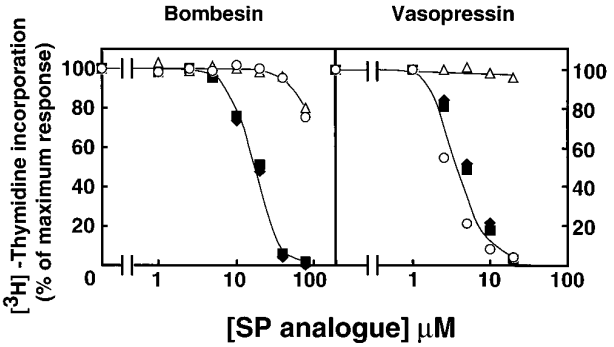

FIG. 6. **Effect of various SP analogues on bombesin- or vasopressin-induced DNA synthesis in Swiss 3T3 cells.** Confluent and quiescent cultures of Swiss 3T3 cells in 33-mm dishes were stimulated with either 0.3 nM bombesin (*left panel*) or 10 nM vasopressin (*right panel*) in the absence or presence of increasing concentrations of various SP analogue compounds: ■, D-Arg-Pro-Lys-Pro-D-Phe-Gln-D-Trp-Phe-D-Trp-Leu-Leu-NH_2; ♦, D-Arg-Pro-Lys-Pro-D-Phe-Gln-D-Trp-Phe-D-Trp-Leu-**Val**-NH_2; △, D-Arg-Pro-Lys-Pro-D-Phe-Gln-D-Trp-Phe-D-Trp-Leu-**Gly**-NH_2; ○, D-Arg-Pro-Lys-Pro-D-Phe-Gln-D-Trp-Phe-D-Trp-Leu-NH_2. All cultures were incubated in the presence of 0.5 μg/ml insulin. After 40 h the incorporation of [^{3}H]thymidine into acid-precipitable material was measured. A representative of three independent experiments is shown. Each point is the mean of duplicate samples expressed as the percentage of maximum DNA synthesis induced by neuropeptide in the absence of SP analogue.

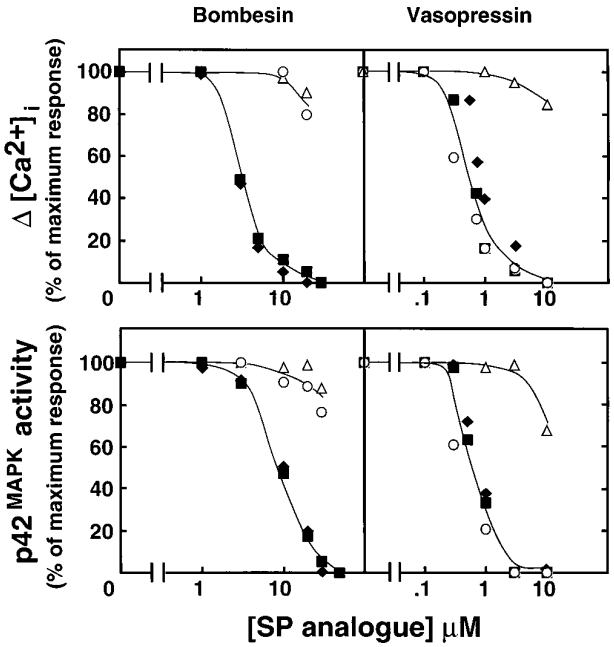

FIG. 7. *Upper*, effect of various SP analogues on bombesin- or vasopressin-induced Ca^{2+} mobilization. Confluent and quiescent cultures of Swiss 3T3 cells loaded with fura-2/AME were stimulated with either 3 nM bombesin (*left panel*) or 10 nM vasopressin (*right panel*) in the absence or presence of increasing concentrations of various SP analogue compounds as indicated in the key to Fig. 6. $[Ca^{2+}]_i$ was measured as described under "Experimental Procedures." *Lower*, effect of various SP analogues on bombesin- or vasopressin-induced MAP kinase activation. Confluent and quiescent cultures of Swiss 3T3 cells in 90-mm dishes were stimulated for 5 min with either 3 nM bombesin (*left panel*) or 10 nM vasopressin (*right panel*) in the absence or presence of increasing concentrations of various SP analogues. The cultures were then lysed and the lysates immunoprecipitated with a polyclonal anti-$p42^{MAPK}$ antibody prior to analysis of MAP kinase activity as described under "Experimental Procedures." A representative of three independent experiments is shown. Each point is the mean of duplicate samples expressed as the percentage of the maximum stimulation induced by either bombesin or vasopressin in the absence of SP analogue.

logue coordinately inhibited vasopressin-induced Ca^{2+} mobilization, 80K/MARCKS phosphorylation, MAP kinase activation, tyrosine phosphorylation, and reinitiation 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^5,D-$Trp^{7,9}$,Leu^{11}]SP could also coordinately 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^5,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^5,D-$Trp^{7,9}$,Leu^{11}]SP and [D-Arg^1,D-Trp^5,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-$Trp^{5,7,9}$,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^5,D-$Trp^{7,9}$,Leu^{11}]SP to form [D-Arg^1,D-Phe^5,D-$Trp^{7,9}$]SP^{1-10} has provided novel mechanistic insight into this problem. We found that [D-Arg^1,D-Phe^5,D-$Trp^{7,9}$]SP^{1-10} potently inhibited vasopressin-induced mitogenesis, Ca^{2+} mobilization, and MAP kinase activation. In contrast, [D-Arg^1,D-Phe^5,D-$Trp^{7,9}$]SP^{1-10} did not significantly block bombesin-induced mitogenesis and only weakly inhibited Ca^{2+} mobilization and MAP kinase activation, even at concentrations where these responses were completely inhibited by either [D-Arg^1,D-Phe^5,D-$Trp^{7,9}$,Leu^{11}]SP or [D-Arg^1,D-Phe^5,D-$Trp^{7,9}$,Val^{11}]SP. Therefore, the SP analogue [D-Arg^1,D-Phe^5,D-$Trp^{7,9}$]SP^{1-10} 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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