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[Leu^{13}-ψ(CH$_2$NH)Leu^{14}]BOMBESIN IS A SPECIFIC BOMBESIN RECEPTOR ANTAGONIST IN SWISS 3T3 CELLS

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SUMMARY: The pseudopeptide [Leu^{13}-ψ(CH$_2$NH)Leu^{14}]bombesin blocks bombesin-stimulated mitogenesis in Swiss 3T3 cells in a competitive and reversible manner, but not that of other mitogens. It inhibits the mobilization of intracellular Ca^{2+} and activation of protein kinase C by bombesin-like peptides. It acts at receptor level, as shown by inhibition of [^{125}I]GRP binding and reduction in cross-linking of the M_r 75-85,000 receptor-associated protein. Thus [Leu^{13}-ψ(CH$_2$NH)Leu^{14}]bombesin is a specific bombesin receptor antagonist in Swiss 3T3 cells which blocks long-term growth promoting effects of bombesin-like peptides.

Bombesin-like peptides are widely distributed in mammals and have diverse functions including neurotransmission in the central nervous system (1,2,3) and stimulation of exocrine secretion in the pancreas and gut (4,5). They are potent mitogens in vitro (6) and have been proposed to act as growth factors for human fetal lung (7) and human small cell lung cancer (8). Peptide antagonists to bombesin could therefore have wide application and may distinguish receptors or mechanisms leading to actions as different as short-term pancreatic secretion and long-term mitogenesis.

The previously described antagonist [DPhe^{12}]bombesin (9) was effective in a pancreatic secretory assay, but not in blocking

Abbreviations: EGF, epidermal growth factor; GRP, gastrin-releasing peptide; IC_{50}, concentration giving 50% inhibition; LψLB, [Leu^{13}-ψ(CH$_2$NH)Leu^{14}]bombesin; Tdr, thymidine.

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bombesin-stimulated DNA synthesis (10). In contrast, the substance P analogues [DArg^{1},DPro^{2},DTrp^{7,9},Leu^{11}]substance P and [DArg^{1},DPhe^{5}, DTrp^{7,9},Leu^{11}]substance P act as bombesin antagonists in mitogenic assays (11,12) but are also antagonists of vasopressin-stimulated DNA synthesis (12-14). The recently described pseudopeptide [Leu^{13}-ψ (CH_2NH)Leu^{14}]bombesin was synthesized during a systematic study of peptide backbone modifications in bombesin analogues (10). It was characterized in guinea pig pancreatic acinar cells, where it caused 50% inhibition (IC_{50}) of bombesin-stimulated amylase release at 35nM. In one assay it also inhibited bombesin-stimulated DNA synthesis in Swiss 3T3 cells. In order to define the activity of [Leu^{13}-ψ(CH_2NH) Leu^{14}]bombesin against the growth-promoting effects of bombesin, we have now characterized its specificity and mechanism of action in Swiss 3T3 cells.

MATERIALS AND METHODS

Culture of Swiss 3T3 cells (15) and assays of DNA synthesis by [^{3}H]thymidine ([^{3}H]Tdr) incorporation (6,15) were as previously described. Receptor binding assays utilized ^{125}I-labelled gastrin-releasing peptide ([^{125}I]GRP) and epidermal growth factor ([^{125}I]EGF) (11,16). Cytosolic [Ca^{2+}] was measured by fluorimetry using the indicator fura-2 (17). The M_r 75-85,000 component of the bombesin receptor was identified by cross-linking of [^{125}I]GRP (18).

Materials: Radiochemicals were obtained from Amersham. Bombesin, GRP, insulin and vasopressin were from Sigma. [DArg^{1},DPhe^{5},DTrp^{7,9}, Leu^{11}]substance P was from Peninsula. [Leu^{13}-ψ(CH_2NH)Leu^{14}]bombesin was prepared as previously described (10). Fura-2^{2} tetraacetoxymethyl ester was from Calbiochem.

RESULTS AND DISCUSSION

Swiss 3T3 cells attain quiescence in serum-depleted medium and can be stimulated to resume cell division by adding defined growth factors, including bombesin and its mammalian homologue, gastrin-releasing peptide, GRP (6). [Leu^{13}-ψ(CH_2NH)Leu^{14}]bombesin markedly increased the concentration of GRP required for half-maximal DNA synthesis from 0.8nM alone, to 4.8nM in the presence of 1μM [Leu^{13}-ψ(CH_2NH)Leu^{14}]bombesin (Fig. 1A). Inhibition of DNA synthesis was

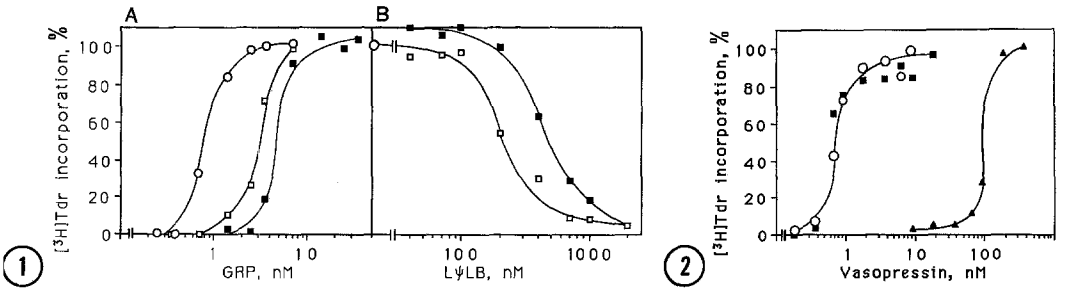

Figure 1 Effects of [Leu^{13}-ψ(CH$_2$NH)Leu^{14}]bombesin (LψLB) on GRP-stimulated mitogenesis.

A. Dose-response curves for GRP-induced DNA synthesis. Quiescent cultures of Swiss 3T3 cells were washed twice, then incubated at 37°C with 1μCi/ml [^{3}H]Tdr, 1μg/ml insulin and various concentrations of GRP in the absence (o) or presence of 500nM (□) or 1μM (■) LψLB. After 40h DNA synthesis was estimated by [^{3}H]Tdr incorporation into acid-precipitable material. Each point represents the mean of 2 determinations (100% = 7.6 x 10^{5}cpm).

B. Dose-response curve for LψLB inhibition of DNA synthesis induced by 2.7nM (□) or 3.6nM (■) GRP in the presence of 1μCi/ml [^{3}H]Tdr and 1μg/ml insulin. Each point represents the mean of 2 determinations (100% = 8.0 x 10^{5}cpm).

Figure 2 Dose-response curves for vasopressin-induced DNA synthesis. Cultures were incubated with 1μCi/ml [^{3}H]Tdr, 1μg/ml insulin and various concentrations of vasopressin in the absence (o) or presence of 1μM LψLB (■) or 20μM [DArg^1,DPhe^5,DTrp^{7,9},Leu^{11}]substance P (▲). Each point represents the mean of 2 determinations (100% = 6.8 10^{5}cpm).

completely overcome by high concentrations of GRP, indicating that the effect is competitive and reversible. The effect of the antagonist was dose dependent (Fig. 1B) with an IC_{50} of 240nM in the presence of 2.7nM GRP. As expected, the antagonist also blocked DNA synthesis stimulated by other bombesin-like peptides including bombesin and litorin.

Bombesin and vasopressin bind to distinct receptors in Swiss 3T3 cells (11,13). Since the substance P antagonists inhibit the stimulation of DNA synthesis by both bombesin and vasopressin (11-14), it was important to test rigorously the specificity of the new antagonist. [Leu^{13}-ψ(CH$_2$NH)Leu^{14}]bombesin did not inhibit mitogenesis stimulated by platelet-derived growth factor, EGF, phorbol 12,13-dibutyrate, cholera toxin or 8-bromoadenosine 3′:5′-cyclic monophosphate. Importantly, it was tested in assays of vasopressin-stimulated mitogenesis at a concentration (1μM) fully effective against GRP, but there was no displacement of the dose-response curve (Fig. 2). In

contrast, a concentration of [DArg^{1},DPhe^{5},DTrp^{7,9},Leu^{11}]substance P (20μM) equipotent against GRP (12) caused marked inhibition of vasopressin-stimulated DNA synthesis. In addition, [Leu^{13}-ψ(CH_2NH)Leu^{14}]bombesin did not interfere with the binding of [^{3}H]vasopressin to Swiss 3T3 cells. Thus [Leu^{13}-ψ(CH_2NH)Leu^{14}]bombesin is both a potent and specific antagonist of bombesin/GRP in Swiss 3T3 cells.

Addition of bombesin or GRP to quiescent fibroblasts leads to an array of early signals which precede DNA synthesis (19). Among these early intracellular signals are transient rises in cytosolic Ca^{2+} concentrations (17) and protein kinase C activation, which mediates inhibition of [^{125}I]EGF binding (16). We have shown that both these signals are blocked by [Leu^{13}-ψ(CH_2NH)Leu^{14}]bombesin (Fig. 3). Inhibition of [^{125}I]EGF binding by GRP (3.6nM) was prevented in a

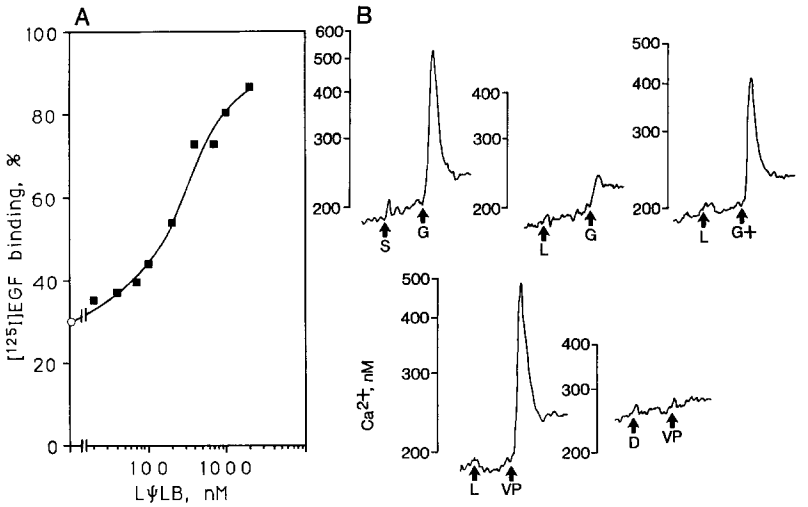

Figure 3 Effects of [Leu^{13}-ψ(CH_2NH)Leu^{14}]bombesin on early cellular responses stimulated by GRP.

A. LψLB reverses the inhibition of [^{125}I]EGF binding induced by GRP. Quiescent Swiss 3T3 cells were incubated for 1h at 37°C in binding medium (11) containing 0.2nM [^{125}I]EGF and 3.6nM GRP alone (o) or in the presence of various concentrations of LψLB (■). Values are expressed as percentages of the specific binding obtained with 0.2nM [^{125}I]EGF alone. Each point represents the mean of 3 determinations.

B. Effect of LψLB on the cytosolic Ca^{2+} changes caused by GRP and vasopressin. Quiescent Swiss 3T3 cells on Cytodex 2 beads (Pharmacia) were washed and incubated for 10 min with 1μM fura-2, then resuspended in electrolyte solution (17) in the fluorimeter and stirred. Fluorescence was recorded continuously while additions were made as indicated: S, electrolyte solution; G, 7.2nM GRP; G+, 72nM GRP; L, 500nM LψLB; VP, 18nM vasopressin: D, 10μM [DArg^{1},DPhe^{5},DTrp^{7,9},Leu^{14}]-substance P.

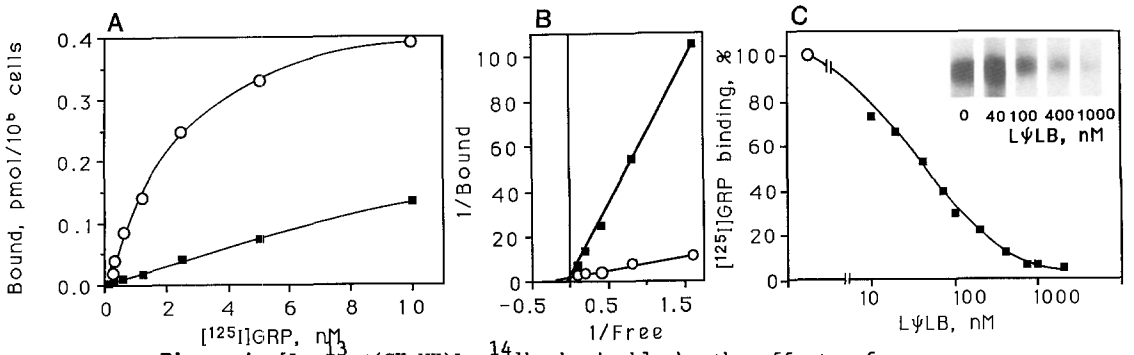

Figure 4 [Leu^{13}-ψ(CH$_2$NH)Leu^{14}]bombesin blocks the effects of bombesin/GRP at the receptor.

A. Concentration-dependence of [^{125}I]GRP binding to Swiss 3T3 cells. Specific cell-associated [^{125}I]GRP binding (11) was measured in quiescent cells incubated for 30 min at 37°C in binding medium containing various concentrations of [^{125}I]GRP in the absence (o) or presence (■) of 500nM LψLB. Each point represents the mean of 2 determinations. Specific binding is expressed in pmol per 10^6 cells.

B. Data from A. shown in a double reciprocal plot.

C. Dose-response curve for LψLB inhibition of [^{125}I]GRP binding to Swiss 3T3 cells. Specific cell-associated [^{125}I]GRP binding was measured in quiescent cells incubated for 30 min at 37°C in binding medium containing 1nM [^{125}I]GRP alone (o) or with various concentrations of LψLB (■). Values are expressed as percentages of binding obtained without antagonist. Each value represents the mean of 3 determinations (100% = 8.4 10^3cpm).

Inset Effect of LψLB on the affinity-labelling of the M_r 75-85,000 bombesin receptor-associated protein. Quiescent cultures of Swiss 3T3 cells were washed with binding medium (18), then incubated at 24°C in binding medium (pH 7.0) containing 1nM [^{125}I]GRP and various concentrations of LψLB. After 10 min they were washed, then incubated at 24°C in binding medium (pH 7.4) containing 6mM ethylene glycol bis (succinimidyl succinate). After 10 min they were washed with cold medium and solubilized in sample buffer, immediately boiled for 5 min, then electrophoresed on a 10% polyacrylamide gel.

concentration-dependent fashion with half-maximal effect at 680nM (Fig. 3A). The increase in cytosolic Ca^{2+} caused by addition of 7.2nM GRP (Fig. 3B) was attenuated by 500nM [Leu^{13}-ψ(CH$_2$NH)Leu^{14}]bombesin, but this effect was overcome by 72nM GRP. In contrast, [Leu^{13}-ψ(CH$_2$NH)Leu^{14}]bombesin did not prevent the Ca^{2+} flux stimulated by 18nM vasopressin, although this could be abolished by 10μM [DArg^1, DPhe^5,DTrp^{7,9},Leu^{11}]substance P.

Bombesin binds to specific, high-affinity binding sites in Swiss 3T3 cells (11). To determine whether [Leu^{13}-ψ(CH$_2$NH)Leu^{14}]bombesin exerts its effects through this receptor, binding measurements and chemical cross-linking studies were undertaken. Figure 4A shows that the antagonist strongly inhibited [^{125}I]GRP binding, and a double

reciprocal plot of these data (Fig. 4B) shows that [Leu^{13}-ψ(CH_2NH) Leu^{14}]bombesin reduced the affinity of the receptors for [^{125}I]GRP, but did not change the number of binding sites. The antagonist inhibited [^{125}I]GRP binding in a dose-dependent manner (Fig. 4C). In addition, cross-linking of [^{125}I]GRP to the recently described M_r 75-85,000 glycoprotein component of the bombesin/GRP receptor (18) was competitively blocked by increasing concentrations of the antagonist.

These results demonstrate that the pseudopeptide [Leu^{13}-ψ(CH_2NH) Leu^{14}]bombesin is a potent and specific antagonist of bombesin-like peptides in Swiss 3T3 cells, which acts at the receptor. It blocks specific binding of [^{125}I]GRP to the receptor, and cross-linking of this ligand to the M_r 75-85,000 receptor-associated glycoprotein. It prevents the early intracellular signals elicited by bombesin and inhibits long-term stimulation of DNA synthesis. Crucially, [Leu^{13}-ψ(CH_2NH)Leu^{14}]bombesin does not affect mitogenesis or cytosolic Ca^{2+} signals induced by other mitogens, including vasopressin. This specific bombesin antagonist will be useful in defining subtypes of receptor in different tissues, and its ability to inhibit bombesin-induced mitogenesis makes it an interesting candidate for studies in small cell lung cancer.

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