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Discovery of a Novel Class of Neuromedin B Receptor Antagonists, Substituted Somatostatin Analogues

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SUMMARY

Bombesin-related peptides have widespread activities in the central nervous system and peripheral tissues. Recent studies show two subtypes of receptors; a gastrin-releasing peptide (GRP) receptor subtype and a neuromedin B (NMB) receptor subtype exist. In contrast to the GRP receptor, no antagonists exist for the NMB receptor. In the present study we report that certain somatostatin (SS) octapeptide analogues function as selective NMB receptor antagonists. The most potent analogue, D-Nal-Cys-Tyr-D-Trp-Lys-Val-Cys-Nal-NH_2, inhibited binding of ^{125}I-[D-Tyr^0]NMB to NMB receptor-transfected 3T3 cells and C6 cells. This analogue had 100-fold lower affinity for GRP receptors. Structure-function studies were performed by synthesizing 18 structurally related SS octapeptide analogues; each of these analogues, but not native SS-14 or SS-28, also inhibited binding to NMB receptors. The stereochemistry at positions 1, 2, 7, and 8, the hydrophobicity and ring size of the substitution in positions 1, 3, and 4, and the basicity of the group in position 5 were all important in determining NMB receptor affinity. No SS octapeptide analogue increased [^{3}H]inositol phosphates in NMB receptor-transfected cells; however, each analogue inhibited NMB-stimulated increases. The most potent analogue, D-Nal-Cys-Tyr-D-Trp-Lys-Val-Cys-Nal-NH_2, caused a parallel rightward shift of the NMB dose-response curve, the Schild plot slope was not significantly different from unity, and the affinity was 230 nM. SS octapeptide analogues also interacted with SS receptors and μ-opioid receptors; however, there was no correlation between the affinities of the analogues for these receptors and their affinities for NMB receptors, demonstrating that these activities can be separated. The results demonstrate for the first time a class of antagonists with >100-fold selectivity for NMB versus GRP receptors. Because the structural requirements for determining NMB, SS, and μ-opioid receptor activity differ, it is likely that highly selective, specific, high affinity NMB receptor antagonists can now be developed that will be useful in defining the role of NMB in various physiological processes.

The mammalian Bn-related peptides GRP and NMB have a wide range of biological and pharmacological effects (1, 2). These include stimulation of the release of numerous gastrointestinal hormones and peptides (3), stimulation of exocrine gland secretion (4), chemotaxis (5), contraction of smooth muscle (6), effects in the central nervous system such as thermoregulation (7), behavioral effects (8), maintenance of circadian rhythm (9), inhibition of thyrotropin release (10), and satiety (11). Bn-related peptides also function as growth factors in numerous normal tissues (bronchial and endometrial stomal cells and 3T3 cells) as well as neoplastic cells such as human small cell lung cancer cells, rat hepatocellular tumor cells, and prostatic and breast adenocarcinoma cells (12-18). Recent structure-function (6, 19-21) and cloning studies (22-25) demonstrate that at least two classes of receptors mediate the actions of Bn-related peptides. One class, the GRP-preferring subtype (GRP receptor), has high affinity for GRP and low affinity for NMB, whereas the other class, the NMB-preferring subtype (NMB-R), has high affinity for NMB and lower affinity for GRP. Both classes of receptors are widely present both in the central nervous system and in the gastrointestinal tract (1, 19-25).

Until recently the physiological importance of Bn-related peptides in mediating various processes, as well as which receptor subtype mediated the various reported biological effects of Bn-related peptides, was unclear. Recently, five different

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ABBREVIATIONS: Bn, bombesin; GRP, gastrin-releasing peptide; cyclo-SS, cyclic somatostatin; cyclo-SS-octa, D-Nal-Cys-Tyr-D-Trp-Lys-Val-Cys-Nal-NH_2; NMB, neuromedin B; NMB-R, neuromedin B receptor; CCK, cholescystokinin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TRH, thyrotropin-releasing hormone; IP, inositol phosphates; SP, substance P; SS, somatostatin; diEt, diethyl; iPr, isopropyl; Nal, naphthylalanine; PYY, peptide YY; DAGO, [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin; BSA, bovine serum albumin; SS-14, somatostatin-14; SS-28, somatostatin-28.

classes of Bn receptor antagonists have been described (21). Members of a number of these classes have high potency, long duration of action, and selectivity for the GRP receptor (21) and thus are useful even *in vivo* for defining the role of GRP or GRP receptors in mediating various physiological events. However, at present no antagonists for the NMB-R that are sufficiently selective or potent have been described (21). Furthermore, when applied to NMB, none of the methodologies used successfully to make potent selective GRP receptor antagonists, such as synthesizing NMB pseudopeptides, des-Met^{10}-NMB, or des-Met^{10}-NMB esters, yielded NMB-R antagonists. Recently it was reported that SS-14 inhibited the cross-linking of ^{125}I-GRP to a 120-kDa protein in Triton extracts of 3T3 cells and human small cell lung cancer cells (26), which are known to possess Bn receptors (25). Recent studies demonstrated that SS-14 also could weakly inhibit binding of opioid receptor ligands to opiate receptors (27, 28), and subsequent structure-function analyses led to the identification of various D-amino acid-substituted and constrained amino acid-substituted cyclo-SS analogues that functioned as potent μ-opioid receptor antagonists (29, 30). Therefore, in the present study we explored the ability of SS-14, SS-28, and various cyclo-SS octapeptide analogues to function as NMB or GRP receptor antagonists. Our results demonstrate that some such SS analogues function as NMB-R antagonists, having >100-fold selectivity for NMB-R over GRP receptors. Furthermore, the structure-function relationships differ for NMB, μ-opioid, and SS receptors, suggesting that it will be possible to develop selective high affinity NMB-R antagonists, as was done for the μ-opioid receptors.

Experimental Procedures

Materials

Rat glioblastoma C6 cells were obtained from the American Type Culture Collection (Rockville, MD), Dulbecco's modified essential medium, fetal bovine serum, and geneticin (aminoglycoside G-418) were from GIBCO (Waltham, MA), and cell culture flasks and 24-well plates were obtained from Costar Co. (Cambridge, MA).

BSA (fraction V) and HEPES were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN); soybean trypsin inhibitor, EGTA, and bacitracin were from Sigma Chemical Co. (St. Louis, MO); glutamine was from the Media Section, National Institutes of Health (Bethesda, MD); NMB, [Tyr^4]Bn, Bn, GRP, and endothelin-1 were from Peninsula Laboratories (Belmont, CA); Na^{125}I was from Amersham Co. (Arlington Heights, IL); *myo*-[2-^{3}H]inositol (16–20 Ci/mmol) was from New England Nuclear (Boston, MA); SS-14 and SS-28 were from Bachem (Torrence, CA); Dowex AG1-X8 anion exchange resin (100–200 mesh, formate form) was from Bio-Rad (Richmond, CA); and Hydro-Fluor scintillation fluid, methanol (absolute), and hydrochloric acid were from the J.T. Baker Chemical Co. (Phillipsburg, NJ).

Methods

Transfection and maintenance of cell lines. As described previously (22), BALB 3T3 cells expressing a stably transfected rat NMB-R (NMB-R-transfected cells) were obtained using calcium phosphate precipitation of a full length NMB-preferring Bn receptor clone generated from rat esophagus and subcloned into a modified version of the pCD2 plasmid. Cells were passaged every 3–4 days at confluence, using 0.1% trypsin in 1 mM EDTA. Rat glioblastoma C6 tumor cells were maintained similarly and were passaged weekly at confluence. Both cell lines were cultured at 37° in a 5% CO_2 atmosphere. Rat AR42J pancratic acinar cells were cultured in Dulbecco's modified medium without antibiotics and supplemented with 10% (v/v) fetal bovine serum. The incubation atmosphere consisted of 10% CO_2/90% humidified air at 37°.

Preparation of rat pancreatic acini. Dispersed acini from guinea pig pancreas were prepared as described previously (31).

Preparation of peptides. Peptides were synthesized on methybenzhydrylamine resin using standard solid-phase procedures and were cleaved with hydrogen fluoride/anisol mixtures. Peptides were cyclized in dilute 90% acetic acid solution by titration with I_2 and were purified by gel filtration on Sephadex G-25 in 50% acetic acid and by gradient elution on C18 silica using acetonitrile/0.1% trifluoroacetic acid buffers. The methods have been described in detail previously (32). Homogeneity was assessed by thin layer chromatography, analytical high performance liquid chromatography, amino acid analysis, and mass spectrometry and was determined to be >96% for each peptide.

Preparation of ^{125}I-[D-Tyr^o]NMB. ^{125}I-[D-Tyr^o]NMB (2200 Ci/mmol) was prepared using Iodo-Gen, as described recently (33). In brief, 0.4 μg of Iodo-Gen was added to 8.0 μg of [D-Tyr^o]NMB with 2 mCi of Na^{125}I, in 20 μl of 0.5 M KH_2PO_4 buffer, pH 7.4. After incubation at 22° for 6 min, 300 μl of 1.5 M dithiothreitol were added and the reaction mixture was incubated at 80° for 60 min. Free ^{125}I was separated by application of the reaction mixture to a Sep-Pak cartridge (Waters Associates, Milford, MA), which had been prepared by washing with 5 ml of methanol, 5 ml of 0.1% trifluoroacetic acid, and 5 ml of water. Free ^{125}I was eluted by 200-μl sequential elutions (10 times) with 60% acetonitrile/0.1% trifluoroacetic acid. Radiolabeled peptide was separated from unlabeled peptide by combining the three elutions with the highest radioactivity and applying them to a high performance liquid chromatograph (Waters Associates model 204, with a Rheodyne injector), using a 0.46- × 25-cm μBondapak reverse phase column. The column was eluted with a linear gradient of acetonitrile and 0.1% trifluoroacetic acid, from 16 to 64% (v/v) acetonitrile in 60 min, with a flow rate of 1.0 ml/min. ^{125}I-[D-Tyr^o]NMB was stored with 1% (w/v) BSA at −20° and was stable for at least 6 weeks.

Binding of ^{125}I-[D-Tyr^o]NMB to C6 glioblastoma and NMB-R-transfected cells. Binding studies using rat glioblastoma C6 cells or NMB-R-transfected cells were performed as described previously (33, 34), by suspending disaggregated cells in binding buffer, which was composed of standard buffer (130 nM NaCl, 7.7 mM KCl, 1.0 mM EGTA, 0.02% soybean trypsin inhibitor) additionally containing 50 mM HEPES, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 2.2 mM KH_2PO_4, 0.015% glutamine, and 0.2% (w/v) BSA, pH 7.4. Incubations contained 75 pM ^{125}I-[D-Tyr^o]NMB and 15 × 10^6 C6 cells/ml or 2 × 10^6 NMB-R-transfected cells/ml, for 60 min at 22°. Nonsaturable binding of ^{125}I-[D-Tyr^o]NMB was the amount of radioactivity associated with C6 cells or NMB-R-transfected cells when the incubation mixture contained 1 μM NMB. Nonsaturable binding was <15% of total binding in all experiments; all values in this paper are reported as saturable binding (i.e., total minus nonsaturable binding).

Binding of ^{125}I-[Tyr^4]Bn to acini. ^{125}I-[Tyr^4]Bn (2000 Ci/mmol) was prepared using the modification (6) of the method described previously (35). ^{125}I-[Tyr^4]Bn was separated from ^{125}I using a Sep-Pak cartridge and was separated from unlabeled peptide by reverse phase high performance liquid chromatography on a column (0.46 × 25 cm) of μBondapak C_{18}. The column was eluted isocratically with acetonitrile (22.5%) and triethylammonium phosphate (0.25 M, pH 3.5) (77.5%), at a flow rate of 1 ml/min. Incubations contained 50 pM ^{125}I-[Tyr^4]Bn and were performed for 60 min at 37° with pancreatic acini. Nonsaturable binding of ^{125}I-[Tyr^4]Bn was the amount of radioactivity associated with the acini when the incubation contained 50 pM ^{125}I-[Tyr^4]Bn plus 1 μM Bn. All values shown are for saturable binding, i.e., binding measured with ^{125}I-[Tyr^4]Bn alone (total) minus binding measured in the presence of 1 μM unlabeled Bn (nonsaturable binding). Nonsaturable binding was <10% of total binding in all experiments.

Membrane receptor assays. Membranes were prepared from rat olfactory bulb (NMB membrane receptor assay), AR42J cells (GRP and SS membrane receptor assays), guinea pig cerebral cortex (neurokinin$_1$, histamine H_1, and σ membrane receptor assays), rat pancreas (CCK_A receptor assay), rat cerebral cortex (CCK_B, PYY, neurotensin, α_1-adrenergic, α_2-adrenergic, muscarinic cholinergic,

neural benzodiazepine, peripheral benzodiazepine, adenosine, calcium channel, and *N*-methyl-D-aspartate membrane receptor assays), A10 smooth muscle cells (Endothelin membrane receptor assay), rat forebrain (TRH, μ-opioid, and δ-opioid membrane receptor assays), rat corpus striatum (dopamine$_1$ and dopamine$_2$ membrane receptor assays), and rat frontal cortex (serotonin$_2$ membrane receptor assay). Membranes were prepared using a Polytron (setting 6, 15 sec) in ice-cold 50 mM Tris·HCl, unless otherwise specified below, and were centrifuged twice at 39,000 × *g* for 10 min, with an intermediate resuspension in fresh buffer. Final pellets were resuspended in 50 mM Tris·HCl containing 0.1 mg/ml bacitracin and 0.1% BSA for the NMB and GRP membrane receptor assays and in 10 mM Tris·HCl for the SS receptor assay. For the NMB and GRP membrane binding assays 50 pM ^{125}I-NMB (or 50 pM ^{125}I-[Tyr^4]Bn) was used in a 30-min incubation at 4°, whereas for the SS assay the incubation was for 25 min at 30° with 50 pM ^{125}I-[Tyr^1]SS in 50 mM HEPES, pH 7.4, with 0.1% BSA, 5 mM $MgCl_2$, 0.02 mg/ml bacitracin, 200 kallikrein-inactivating units/ml trasylol, and 0.02 mg/ml phenylmethylsulfonyl fluoride. Incubations were terminated by rapid filtration through GF/B filters that had been presoaked in 0.1% polyethyleneimine (NMB and GRP receptor assays). Each filter was washed three times with 5-ml aliquots of ice-cold buffer. The ligands used for the various membrane binding assays were 0.5 nM [^{3}H]SP (neurokinin$_1$ receptor), 0.05 nM ^{125}I-endothelin-1 (endothelin$_A$ receptor), 0.05 nM ^{125}I-CCK-8 (CCK_A and CCK_B receptors), 0.05 nM ^{125}I-PYY (PYY receptor), 2 nM [^{3}H]neurotensin (neurotensin receptor), 1 nM [^{3}H]bradykinin (bradykinin$_2$ receptor), 2 nM [^{3}H]3-methylhistidine-TRH (TRH receptor), 0.4 nM [^{3}H]prazosin (α_1-adrenergic receptor), 0.5 nM [^{3}H]clonidine (α_2-adrenergic receptor), 0.5 nM [^{3}H]-dihydroalprenolol (β_1-adrenergic receptor), 0.05 nM [^{3}H]quinuclidinyl benzilate (muscarinic cholinergic receptor), 0.5 nM [^{3}H]RO15–1788 (neural benzodiazepine receptor), 0.5 nM [^{3}H]RO5–4864 (peripheral benzodiazepine receptor), 0.3 nM [^{3}H]SCH23390 (dopamine$_1$ receptor), 0.25 nM [^{3}H]spiperone (dopamine$_2$ receptor), 0.5 nM [^{3}H]ketanserin (serotonin$_2$ receptor), 1 nM [^{3}H]pyrilamine (histamine H_1 receptor), 1.5 nM [^{3}H]cylcohexyladenosine (adenosine$_1$ receptor), 5 nM [^{3}H]MK-801 (*N*-methyl-D-aspartate receptor), 0.5 nM (+)-[^{3}H]pentazocine (σ receptor), 0.5 nM [^{3}H]DAGO (μ-opioid receptor), and 1 nM [^{3}H][D-Pen,D-Pen]-enkephalin (δ-opioid receptor).

Measurement of phosphoinositides. Total phosphoinositides in C6 and in NMB-R-transfected cells were determined as described previously, with minor modifications (33, 34). Cells were grown to confluence in 24-well plates and then loaded with 100 μCi/ml *myo*-[2-^{3}H]inositol in Dulbecco's modified essential medium with 2% fetal bovine serum, at 37° for 48 hr. Cells were washed, incubated in phosphoinositide buffer (standard buffer additionally containing 10 mM LiCl, 20 mM HEPES, 2 mM $CaCl_2$, 2% BSA, and 1.2 mM $MgSO_4$) for 15 min, and then incubated for 60 min at 37° with agonists at various concentrations or with 3 nM NMB (a half-maximally effective concentration) and possible antagonists at different concentrations. Reactions were halted using ice-cold 1% HCl in methanol, and the IP were isolated as described previously (15). Briefly, after loading of the anion exchange column, free [^{3}H]glycerophosphorylinositol was removed by washing with 5 mM disodium tetraborate in 60 mM sodium formate. Total [^{3}H]IP were then eluted using 100 mM formic acid in 1.0 M ammonium formate, as described previously (33, 34).

Results

To investigate the ability of SS-14, SS-28, and the various cyclo-SS octapeptide analogues to interact with NMB and GRP receptors, the ability of each to inhibit binding of either ^{125}I-[D-Tyr^0]NMB to NMB-R-transfected cells (Fig. 1) or ^{125}I-[Tyr^4]Bn to GRP receptors on rat pancreatic acini was determined. At 10 μM, SS-14 and SS-28 caused no inhibition of binding of ^{125}I-[Tyr^0]NMB to NMB-R-transfected cells; however, each of the cyclo-SS octapeptide analogues caused significant inhibition of binding of ^{125}I-[Tyr^0]NMB to these cells

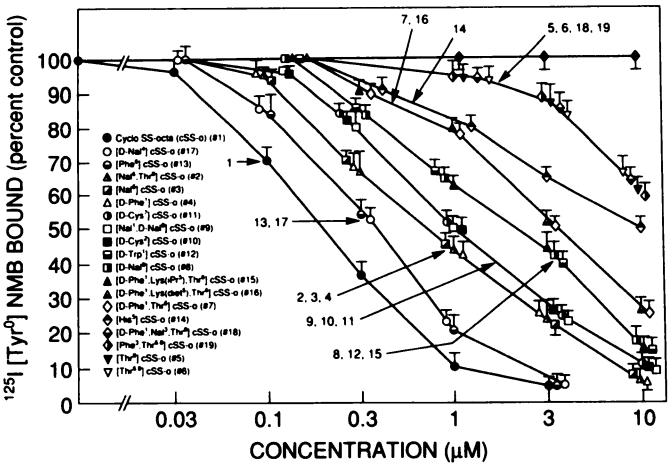

Fig. 1. Ability of various SS octapeptide analogues to inhibit binding of ^{125}I-[D-Tyr^0]NMB to NMB-R on NMB-R-transfected cells. NMB-R-transfected cells (2 × 10^6/ml) were incubated with 75 pM ^{125}I-[D-Tyr^0]NMB and with SS-14, SS-28, or the indicated SS analogue for 30 min at 37°. Results are expressed as the percentage of saturable binding with unlabeled peptide added. Results are means ± 1 SE from four experiments, and in each experiment each value was determined in duplicate. ◆, SS-14 and SS-28.

(Fig. 1). Cyclo-SS-octa (**1**, Table 1) was the most potent, causing detectable inhibition of binding of ^{125}I-[Tyr^0]NMB at 0.1 μM, half-maximal inhibition at 216 nM, and complete inhibition at 3 μM (Fig. 1; Table 1). Cyclo-SS-octa was 2-fold more potent than [Phe^6]-cyclo-SS-octa (**13**, Table 1) and [D-Nal^4]-cyclo-SS-octa (**17**, Table 1) (K_i, 400 nM; Table 1), which were equipotent; 4-fold more potent than [Nal^6,Thr^8]-cyclo-SS-octa, [Nal^6]-cyclo-SS-octa, and [D-Phe^1]-cyclo-SS-octa (**2-4**, Table 1; K_i, 700–800 nM) (Fig. 1); 6-fold more potent than [Nal^1,D-Nal^8]-cyclo-SS-octa, [D-Cys^2]-cyclo-SS-octa, and [D-Cys^7]-cyclo-SS-octa (**9-11**, Table 1; K_i, 1–1.2 μM) (Fig. 1); 9-fold more potent than [D-Nal^8]-cyclo-SS-octa, [D-Trp^1]-cyclo-SS-octa, and [D-Phe^1,Lys(iPr)^5,Thr^6]-cyclo-SS-octa (**8**, **12**, and **15**, Table 1; K_i, 1.4–2.3 μM); 18-fold more potent than [D-Phe^1,Lys(diEt)^5,Thr^6]-cyclo-SS-octa (**16**, Table 1; K_i, 3.9 μM); 45-fold more potent than [His^5]-cyclo-SS-octa (**14**, Table 1; K_i, 9.9 μM); and 69-fold more potent than [Thr^8]-cyclo-SS-octa, [Thr^{6,8}]-cyclo-SS-octa, [Phe^3,Thr^{6,8}]-cyclo-SS-octa, and [D-Phe^1,Nal^3,Thr^8]-cyclo-SS-octa (**5**, **6**, **18**, and **19**, Table 1; K_i, 14–19 μM). In contrast, but similarly to SS-14 and SS-28, 13 of the cyclo-SS octapeptides caused no inhibition of ^{125}I-[Tyr^4]Bn binding to GRP receptors on rat pancreatic acini, and the remaining six analogues had very low affinity for this receptor, with each having an affinity of >15 μM (Table 1). The three most potent analogues, cyclo-SS-octa, [Phe^6]-cyclo-SS-octa, and [D-Nal^4]-cyclo-SS-octa (**1**, **13**, and **17**, Table 1) had 84-, >100-, and >100-fold greater affinity, respectively, for the NMB-R than the GRP receptor (Table 1).

To determine whether SS-14, SS-28, or the various cyclo-SS octapeptide analogues functioned as agonists or antagonists at the Bn receptor subtypes, their ability (at 10 μM) to stimulate increases in [^{3}H]IP in NMB-R-transfected cells or to stimulate amylase release or inhibit Bn-stimulated amylase release from rat pancreatic acini possessing GRP receptors was assessed (Table 2). Neither SS-14, SS-28, nor any of the 19 SS octapeptide analogues at a concentration of 10 μM had agonist activity and stimulated increases in [^{3}H]IP in NMB-R-transfected cells or amylase release from rat pancreatic acini, which have GRP

TABLE 1

Affinity of SS-14, SS-28, and various SS octapeptide analogues for NMB-R on C6 cells or transfected BALB 3T3 cells or GRP receptors on rat pancreatic acini

Analogue	Peptide	NMB-R[a]			GRP receptor, rat pancreatic acini	
		C6 cells, K_i, ^{125}I-NMB binding	Transfected cells: IC_{50}, NMB-stimulated [^{3}H]IP	Transfected cells: K_i, ^{125}I-NMB binding	IC_{50}, amylase release	K_i, ^{125}I-[Tyr^4]Bn binding
		nM	nM	nM	nM	nM
1	Cyclo-SS-octa	59 ± 9	885 ± 98	216 ± 36	No activity at 10 μM	18,264 ± 2,110
2	[Nal^6,Thr^8]-cyclo-SS-octa	226 ± 36	6,673 ± 435	772 ± 94	No activity at 10 μM	16,291 ± 3,818
3	[Nal^6]-cyclo-SS-octa	997 ± 76	2,880 ± 188	697 ± 64	No activity at 10 μM	No activity at 10 μM
4	[D-Phe^1]-cyclo-SS-octa	848 ± 191	3,757 ± 568	818 ± 68	No activity at 10 μM	21,947 ± 4,265
5	[Thr^8]-cyclo-SS-octa	3,792 ± 1,084	No activity at 10 μM	14,766 ± 2,651	No activity at 10 μM	No activity at 10 μM
6	[Thr^{6,8}]-cyclo-SS-octa	8,286 ± 2,427	No activity at 10 μM	16,398 ± 4,455	No activity at 10 μM	No activity at 10 μM
7	[D-Phe^1,Thr^8]-cyclo-SS-octa	1,452 ± 78	36,235 ± 4,974	4,362 ± 328	No activity at 10 μM	30,981 ± 4,653
8	[D-Nal^8]-cyclo-SS-octa	670 ± 73	5,187 ± 987	1,924 ± 201	No activity at 10 μM	No activity at 10 μM
9	[Nal^1,D-Nal^8]-cyclo-SS-octa	1,159 ± 214	4,413 ± 451	1,156 ± 229	No activity at 10 μM	No activity at 10 μM
10	[D-Cys^2]-cyclo-SS-octa	1,147 ± 518	3,992 ± 781	960 ± 109	No activity at 10 μM	No activity at 10 μM
11	[D-Cys^7]-cyclo-SS-octa	1,740 ± 345	1,427 ± 119	1,077 ± 199	No activity at 10 μM	No activity at 10 μM
12	[D-Trp^1]-cyclo-SS-octa	1,778 ± 109	4,688 ± 927	1,411 ± 127	No activity at 10 μM	No activity at 10 μM
13	[Phe^6]-cyclo-SS-octa	213 ± 13	1,173 ± 114	397 ± 72	No activity at 10 μM	No activity at 10 μM
14	[His^5]-cyclo-SS-octa	4,944 ± 930	11,865 ± 1,835	9,863 ± 1,294	No activity at 10 μM	No activity at 10 μM
15	[D-Phe^1,Lys(iPr^5)Thr^6]-cyclo-SS-octa	1,142 ± 105	9,897 ± 2,312	2,328 ± 397	No activity at 10 μM	38,127 ± 21,549
16	[D-Phe^1,Lys(diEt^5)Thr^6]-cyclo-SS-octa	1,089 ± 38	7,212 ± 2,795	3,951 ± 509	No activity at 10 μM	No activity at 10 μM
17	[D-Nal^4]-cyclo-SS-octa	313 ± 33	1,779 ± 295	399 ± 68	No activity at 10 μM	No activity at 10 μM
18	[D-Phe^1,Nal^3,Thr^8]-cyclo-SS-octa	8,322 ± 957	>10 μM	19,816 ± 4,235	No activity at 10 μM	61,637 ± 21,512
19	[Phe^3,Thr^{6,8}]-cyclo-SS-octa	8,485 ± 1,165	>10 μM	14,341 ± 1,819	No activity at 10 μM	No activity at 10 μM
	SS-14	No activity at 10 μM	No activity at 10 μM	No activity at 10 μM	No activity at 10 μM	No activity at 10 μM
	SS-28	No activity at 10 μM	No activity at 10 μM	No activity at 10 μM	No activity at 10 μM	No activity at 10 μM

[a] IC_{50}, concentration causing half-maximal inhibition of the indicated agonist; K_i, affinity of the indicated peptide for the indicated receptor, calculated by the method of Cheng and Prusoff (36). No activity at 10 μM, no agonist or antagonist activity at concentrations up to 10 μM.

receptors (Table 2). Similarly, none of these peptides at this concentration altered the increase in amylase release caused by 0.3 nM Bn in rat pancreatic acini (Table 2). Whereas SS-14, SS-28, and three cyclo-SS octapeptide analogues (**5**, **6**, and **18**, Table 2) had no effect on the 14-fold increase in [^{3}H]IP caused by 3 nM NMB in NMB-R-transfected cells, 16 of the cyclo-SS octapeptide analogues caused some inhibition (Table 2). Five analogues at 10 μM (**1**, **10**, **11**, **14**, and **17**, Table 2) completely inhibited the NMB-stimulated increase in [^{3}H]IP.

To determine the relative abilities of the SS octapeptide analogues to inhibit NMB-stimulated increases in [^{3}H]IP in NMB-R-transfected cells, dose-inhibition curves were determined for each analogue (Fig. 2). Cyclo-SS-octa (**1**, Table 2) was the most potent, causing detectible inhibition at 0.3 μM, half-maximal inhibition at 885 nM, and complete inhibition at 10 μM (Fig. 2). The relative potencies were cyclo-SS-octa (IC_{50}, 885 nM) > [D-Cys^7]-cyclo-SS-octa, [Phe^6]-cyclo-SS-octa, and [D-Nal^4]-cyclo-SS-octa (**11**, **13**, and **17**, Table 1; IC_{50}, 1.2–1.8 μM) > [Nal^6,Thr^8]-cyclo-SS-octa, [Nal^6]-cyclo-SS-octa, and [D-Phe^1]-cyclo-SS-octa (**2**-**4**, Table 1; IC_{50}, 3–6.6 μM) > [D-Nal^8]-cyclo-SS-octa, [Nal^1,D-Nal^8]-cyclo-SS-octa, [D-Cys^2]-cyclo-SS-octa, and [D-Trp^1]-cyclo-SS-octa (**8**-**10** and **12**, Table 1; IC_{50}, 4.4–5.2 μM) > [D-Phe^1,Lys(iPr)^5,Thr^6]-cyclo-SS-octa and [D-Phe^1,Lys(diEt)^5,Thr^6]-cyclo-SS-octa (**15** and **16**, Table 1; IC_{50}, 7.2–9.8 μM) > [His^5]-cyclo-SS-octa (**14**, Table 1; IC_{50}, 11.8 μM) > [D-Phe^1,Nal^3,Thr^8]-cyclo-SS-octa and [Phe^3,Thr^{6,8}]-cyclo-SS-octa (**18** and **19**, Table 1; IC_{50}, >10 μM) > SS-14, SS-28, [Thr^8]-cyclo-SS-octa, and [Thr^{6,8}]-cyclo-SS-octa (no activity at 10 μM). There was a close correlation between the relative abilities of the different SS octapeptide analogues to occupy the NMB-R and inhibit binding of ^{125}I-[D-Tyr^0]NMB to NMB-R-transfected cells and their abilities to inhibit NMB-stimulated increases in [^{3}H]IP in these cells (r = 0.77, p = 0.0001) (Figs. 1 and 2; Table 1).

The 18 cyclo-SS octapeptide analogues of cyclo-SS-octa were made to explore the importance of the different amino acid substitutions in cyclo-SS-octa (**1**, Tables 1 and 2) in determining its ability to function as a NMB-R antagonist. Analogues **8**-**11** (Tables 1 and 2) explored the importance of stereochemistry at positions 1, 2, 7, and 8 of cyclo-SS-octa. Changing Cys^1 or Cys^7 to D-cysteine had equivalent effects, with both substitutions decreasing affinity 5-fold (compare **1**, **10**, and **11**, Table 1). Similarly, insertion of D-Nal in position 8 caused a 6-fold decrease in affinity (compare **1** and **9**, Table 1), and the further addition of Nal^1 for D-Nal^1 did not decrease affinity further (compare **1**, **8**, and **9**, Table 1). The importance of the hydrophobicity and ring size of the substituted amino acid was explored for position 1 (analogues **4**, **6**, **7**, and **12**, Table 1), position 3 (**18** and **19**, Table 1), and position 4 (**17**, Table 1). The insertion of a less hydrophobic group with a different ring size, i.e., D-Phe^1 or D-Trp^1, had only a moderate effect, decreasing potency 4–7-fold (compare **1**, **4**, **6**, **7**, and **12**, Table 1). In contrast, the insertion of more hydrophobic groups, i.e., Nal^3 or D-Phe^3, for Tyr^3 had almost no effect on affinity (compare **5** with **18** and **19**, Table 1), in that when they were added to an analogue with a Thr^8 replacement (**5**, Table 1) no change in affinity occurred. Similarly, the insertion of the more hydrophobic group D-Nal for D-tryptophan in position 4 (compare **1** and **17**, Table 1) had almost no effect on affinity. The importance of the valine substitution in position 6 of cyclo-SS-octa

TABLE 2

Ability of SS-14, SS-28, and related octapeptide analogues to stimulate or alter NMB-stimulated increases in [^{3}H]IP in NMB-R-transfected cells or amylase release in rat pancreatic acini

Rat pancreatic acini or *myo*-[2-^{3}H]inositol-loaded NMB-R-transfected cells were incubated with either no additives, SS octapeptide analogue, Bn, or NMB, or a combination, for 30 min at 37°. Amylase release from pancreatic acini was expressed as the indicated percentage of the total cellular amylase released during the incubation. To test for inhibitory effects, the effects of SS analogues (10 μM) were determined on 0.3 nM Bn-stimulated amylase release or 3 nM NMB-stimulated increase in [^{3}H]IP, which are half-maximally effective agonist concentrations. Results are means ± SE from at least four separate experiments, and in each experiment each value was determined in duplicate.

Analogue	SS analogue added		NMB-R-transfected cells, [^{3}H]IP		Rat pancreatic acini, amylase release	
	Name	Structure	Alone (10 μM)	+NMB (3 nM)	Alone (10 μM)	+Bn (0.3 nM)
			dpm × 10^3		% *of total*	
	No addition	No addition	13 ± 3	186 ± 58	4 ± 1	15 ± 1
1	Cyclo-SS-octa	D-Nal-Cys-Tyr-D-Trp-Lys-Val-Cys-Nal-NH_2	12 ± 2	8 ± 4*	5 ± 1	15 ± 2
2	[Nal^6,Thr^8]-cyclo-SS-octa	D-Nal-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH_2	11 ± 2	39 ± 7*	5 ± 1	13 ± 1
3	[Nal^6]-cyclo-SS-octa	D-Nal-Cys-Tyr-D-Trp-Lys-Val-Cys-Nal-NH_2	11 ± 1	65 ± 15*	3 ± 1	15 ± 1
4	[D-Phe^1]-cyclo-SS-octa	D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Nal-NH_2	13 ± 1	33 ± 4*	4 ± 2	14 ± 1
5	[Thr^8]-cyclo-SS-octa	D-Nal-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH_2	11 ± 2	179 ± 13	3 ± 1	12 ± 1
6	[Thr^{6,8}]-cyclo-SS-octa	D-Nal-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH_2	11 ± 2	188 ± 30	3 ± 1	14 ± 2
7	[D-Phe^1,Thr^6]-cyclo-SS-octa	D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Nal-NH_2	15 ± 1	99 ± 24*	3 ± 1	16 ± 1
8	[D-Nal^8]-cyclo-SS-octa	D-Nal-Cys-Tyr-D-Trp-Lys-Val-Cys-D-Nal-NH_2	10 ± 3	67 ± 13*	2 ± 1	14 ± 1
9	[Nal^1,D-Nal^8]-cyclo-SS-octa	Nal-Cys-Tyr-D-Trp-Lys-Val-Cys-D-Nal-NH_2	8 ± 2	28 ± 6*	3 ± 1	16 ± 1
10	[D-Cys^2]-cyclo-SS-octa	D-Nal-D-Cys-Tyr-D-Trp-Lys-Val-Cys-Nal-NH_2	10 ± 3	4 ± 2*	3 ± 1	16 ± 1
11	[D--Cys^7]-cyclo-SS-octa	D-Nal-Cys-Tyr-D-Trp-Lys-Val-D-Cys-Nal-NH_2	10 ± 1	7 ± 4*	2 ± 1	14 ± 1
12	[D-Trp^1]-cyclo-SS-octa	D-Trp-Cys-Tyr-D-Trp-Lys-Val-Cys-Nal-NH_2	9 ± 2	43 ± 2	3 ± 1	13 ± 1
13	[Phe^6]-cyclo-SS-octa	D-Nal-Cys-Tyr-D-Trp-Lys-Phe-Cys-Nal-NH_2	10 ± 1	9 ± 4*	3 ± 1	14 ± 1
14	[His^5]-cyclo-SS-octa	D-Nal-Cys-Tyr-D-Trp-His-Val-Cys-Nal-NH_2	8 ± 1	97 ± 7*	2 ± 1	13 ± 1
15	[D-Phe^1,Lys(iPr^5),Thr^6]-cyclo-SS-octa	D-Phe-Cys-Tyr-D-Trp-Lys(iPr)-Thr-Cys-Nal-NH_2	11 ± 1	67 ± 7*	4 ± 2	13 ± 2
16	[D-Phe^1,Lys(diEt^5),Thr^6]-cyclo-SS-octa	D-Phe-Cys-Tyr-D-Trp-Lys(diEt)-Thr-Cys-Nal-NH_2	11 ± 2	73 ± 15*	5 ± 1	12 ± 1
17	[D-Nal^4]-cyclo-SS-octa	D-Nal-Cys-Tyr-D-Nal-Lys-Val-Cys-Nal-NH_2	12 ± 4	15 ± 2*	4 ± 2	19 ± 1
18	[D-Phe^1,Nal^3,Thr^8]-cyclo-SS-octa	D-Phe-Cys-Nal-D-Trp-Lys-Val-Cys-Thr-NH_2	11 ± 1	143 ± 11	3 ± 1	14 ± 2
19	[Phe^3,Thr^{6,8}]-cyclo-SS-octa	D-Nal-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-NH_2	10 ± 1	119 ± 4	3 ± 1	14 ± 1
	SS-14	Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys	14 ± 1	182 ± 24	3 ± 1	12 ± 1
	SS-28	Ser-Ala-Asn-Ser-Asn-Pro-Ala-Met-Ala-Pro-Arg-Glu-Arg-Lys-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys	12 ± 1	177 ± 13	4 ± 1	10 ± 1

* $p < 0.05$, compared with value with no SS analogue added.

was examined in analogues **3** and **13** (Table 1). Substitution of either Phe^6 (**13**, Table 1) or Nal^6 (**3**, Table 1) caused only a minimal (2–3-fold) decrease in affinity. The insertion of Thr^8 in a position similar to that used in some high affinity SS or μ receptor agonists (33–36) caused a dramatic (80-fold) decrease in NMB-R affinity (compare **1** and **5**, Table 1), whereas insertion of Nal for Val^6 compensated for the Thr^8 substitution and resulted in only a 4-fold decrease (compare **1**, **2**, and **5**, Table 1). The substitution of a less basic group, histidine, for lysine in position 5 caused a marked 50-fold decrease in affinity (compare **1** and **4**, Table 1). Similarly, altering the availability of the primary amino group on Lys^3 by formation of Lys(diEt)^3 or Lys(iPr)^3 caused a >10-fold decrease in affinity for the NMB-R (compare **1**, **15**, and **16**; Table 1).

To investigate further the inhibitory action of the most potent analogue, cyclo-SS-octa (**1**, Tables 1 and 2), its ability to affect the dose-response curve for NMB-stimulated increases in [^{3}H]IP in NMB-R-transfected cells (Fig. 3) or the dose-inhibition curve for NMB inhibition of binding of ^{125}I-[D-Tyr^0]NMB (Fig. 4) to these cells was determined. The addition of 1, 3, or 10 μM cyclo-SS-octa caused a parallel rightward shift in the dose-response curve for NMB-stimulated increases in [^{3}H]IP, with no change in the maximal increase if sufficiently high concentrations of NMB were used (Fig. 3). The magnitude of the rightward shift was proportional to the concentration of cyclo-SS-octa and the concentration of NMB used (Fig. 3). Plotting these data in the form of Schild (Fig. 3, *inset*) gave a regression equation of $y = (1.1 \pm 0.2) \times 7.3$, with a correlation coefficient of 0.8 ($p < 0.001$), and the slope was not significantly different from unity. Calculation of the affinity of cyclo-SS-octa for the NMB-R from these data gave an affinity of 231 ± 43 nM. Analysis of the ability of 0.5 μM cyclo-SS-octa to affect the dose-response curve for the ability of NMB to inhibit binding of ^{125}I-[D-Tyr^0]NMB to NMB-R-transfected cells, using a nonlinear least-squares curve-fitting program, demonstrated that cyclo-SS-octa was functioning as a competitive antagonist (Fig. 4). Specifically, 0.5 μM cyclo-SS-octa caused a decrease in the affinity of NMB for NMB-R (without cyclo-SS-octa, $K_d = 3.1 \pm 0.2$ nM; with 0.5 μM cyclo-SS-octa present, $K_d = 6.0 \pm 0.5$ nM, $p < 0.01$). In contrast, there was no change in the total number of NMB binding sites in the presence of 0.5 μM cyclo-SS-octa (without cyclo-SS-octa, 7.6 ± 0.5 pmol/mg of protein; with 0.5 μM cyclo-SS-octa, 6.6 ± 0.5 pmol/mg of protein).

To investigate the specificity of the inhibitory effects of the cyclo-SS octapeptide analogues, the ability of a number of these analogues to inhibit binding of a number of different ligands for different receptors was determined, as was their ability to interact with NMB-R on C6 glioblastoma cells and to alter biological responses in these cells. C6 glioblastoma cells have

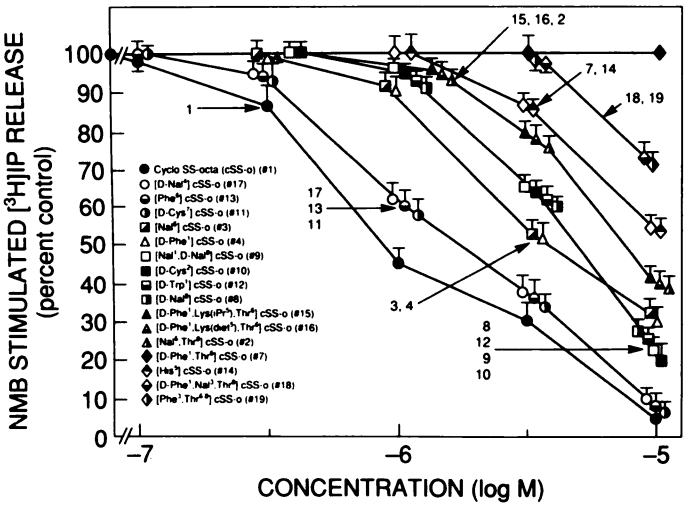

Fig. 2. Ability of SS-14, SS-28, and various cyclo-SS octapeptide analogues to inhibit NMB-stimulated increases in [^{3}H]IP in NMB-R-transfected cells. NMB-R-transfected cells were loaded with 100 μCi/nl *myo*-[2-^{3}H]inositol for 48 hr, washed, and resuspended in phosphoinositide buffer containing 10 mM LiCl, as outlined in Experimental Procedures. Cells were then incubated with 3 nM NMB, a half-maximally effective concentration, alone or in combination with the indicated concentrations of SS analogues for 60 min at 37°. Results are expressed as the percentage of stimulation produced by 3 nM NMB alone. Control and 3 nM NMB-stimulated values were 11,000 ± 400 dpm and 187,000 ± 19,000 dpm (mean ± 1 SE), respectively, from 12 separate experiments, and in each experiment each value was determined in duplicate. ♦, SS-14, SS-28, [Thr^{6,8}]-cyclo-SS-octa (**6**, Tables 1 and 2), and [Thr^8]-cyclo-SS-octa (**5**, Tables 1 and 2).

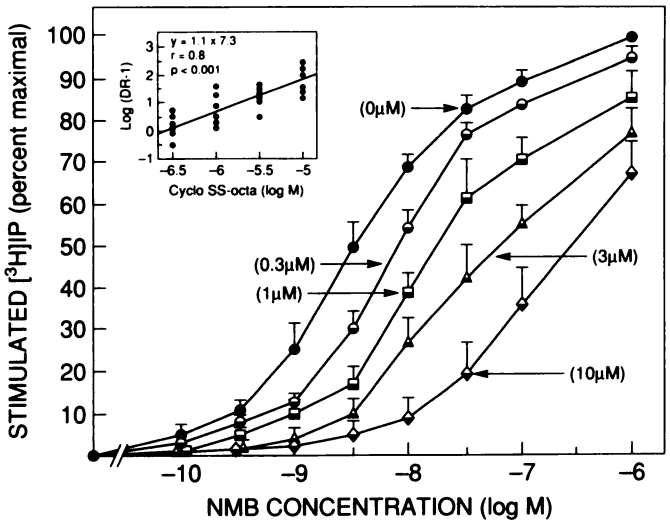

Fig. 3. Effect of increasing concentrations of cyclo-SS-octa (**1**, Tables 1 and 2) on the dose-response curve for NMB-stimulated increases in [^{3}H]IP in NMB-R-transfected cells. NMB-R-transfected cells were incubated with the indicated concentrations of NMB or cyclo-SS-octa for 60 min at 37°, and changes in [^{3}H]IP were determined as described in Experimental Procedures. Results are expressed as the percentage of the increase caused by a maximally effective concentration of NMB, i.e., 1 μM NMB. In these experiments, control and 1 μM NMB-stimulated increases in [^{3}H]IP were 12,300 ± 2,300 dpm and 211,410 ± 40,000 dpm (mean ± 1 SE), respectively. Each value is the mean ± 1 SE from six experiments, and in each experiment each value was determined in duplicate. *Inset*, data plotted in the form of Schild. Regression equation was calculated by least-squares analysis.

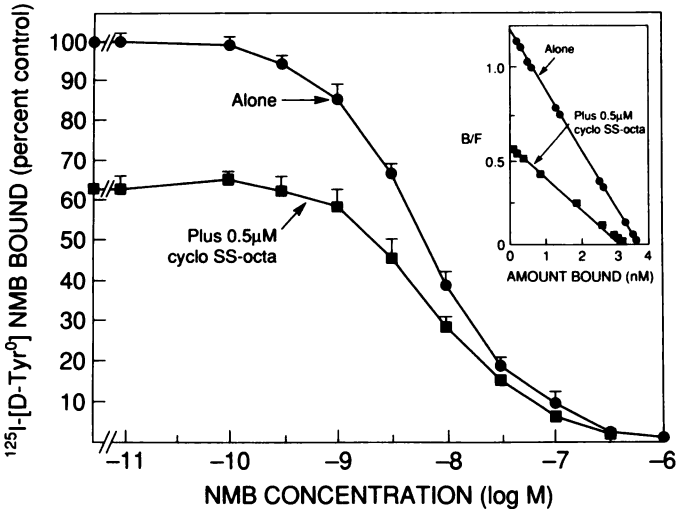

Fig. 4. Effect of a fixed concentration of cyclo-SS-octa (**1**, Tables 1 and 2) on the dose-inhibition curve for NMB inhibition of ^{125}I-[D-Tyr^0]NMB binding to NMB-R-transfected cells. NMB-R-transfected cells were incubated with 75 pM ^{125}I-[D-Tyr^0]NMB, with or without 0.5 μM cyclo-SS-octa and the indicated concentrations of NMB, for 30 min at 37°. Results are expressed as the percentage of the saturable binding seen with no cyclo-SS-octa or unlabeled NMB added. Results are means ± 1 SE from four experiments, and in each experiment each value was determined in duplicate. *Inset*, data plotted as a Scatchard plot after analysis of the NMB dose-inhibition curves using a least-squares curve-fitting program (LIGAND).

been shown to possess NMB-R (37), and therefore the ability of SS octapeptide analogues to interact with native receptors on these cells was compared with their ability to interact with NMB-R on NMB-R-transfected cells. As demonstrated in Table 1, SS-14 and SS-28 did not inhibit binding of ^{125}I-[D-Tyr^0]NMB to C6 cells, and cyclo-SS-octa was the most potent SS octapeptide analogue, having an affinity of 59 ± 9 nM. The affinities of the other 18 SS octapeptide analogues were, in general, in good agreement with those seen for the NMB-R on NMB-R-transfected cells (Table 1). The specificity of the inhibitory actions of two of the most potent analogues, cyclo-SS-octa (**1**, Table 1) and [Nal^6,Thr^8]-cyclo-SS-octa (**2**, Table 1), was demonstrated on C6 glioblastoma cells, inasmuch as each of these SS octapeptide analogues inhibited NMB-stimulated increases in [^{3}H]IP in these cells but had no effect on endothelin-1-stimulated increases in [^{3}H]IP (Table 3). Furthermore, the most potent SS octapeptide analogue, cyclo-SS-octa (**1**, Tables 1 and 2), at 1 μM, a concentration that inhibited binding to NMB-R on rat olfactory bulb membranes by >95%, did not inhibit binding to CCK_A, CCK_B, $endothelin_A$, PYY, $bradykinin_1$, TRH, α_1- or α_2-adrenergic, β_1-adrenergic, muscarinic cholinergic, neural or peripheral benzodiazepine, $dopamine_2$, histamine H_1, $adenosine_1$, σ- or δ-opioid, or *N*-methyl-D-aspartate receptors on plasma membranes in various tissues, determined as described in Experimental Procedures (data not shown).

Cyclo-SS-octa did inhibit binding of [^{3}H]DAGO to μ-opioid receptors on rat forebrain membranes with an affinity of 430 ± 126 nM (Table 4) and inhibited binding of ^{125}I-Bolton-Hunter-CCK-8 to CCK_A receptors on rat pancreatic membranes with an affinity of 5537 ± 7 nM. In previous studies various SS analogues have been reported to have high affinity for μ-opioid receptors as well as SS receptors (29, 30). To compare the ability of SS-14, SS-28, and the various SS octapeptide analogues to interact with both subtypes of Bn recep-

TABLE 3

Ability of two cyclo-SS octapeptide analogues to inhibit NMB- and endothelin-stimulated increases in IP in C6 glioblastoma cells

C6 glioblastoma cells (50,000 cells/well) were incubated with *myo*-[2-^{3}H]inositol for 2 days, washed, and then incubated with or without the indicated peptides for 60 min at 37°, in phosphoinositide buffer containing 10 mM LiCl, as described in Experimental Procedures. [^{3}H]IP were measured using Dowex AG1-X8 anion exchange chromatography, as described in Experimental Procedures. Results are means ± standard errors from three experiments, and in each experiment each value was determined in duplicate.

Peptide added	[^{3}H]IP		
	Alone	+[Nal^6,Thr^8]-cyclo-SS-octa (10 μM)	+Cyclo-SS-octa (10 μM)
	dpm × 10^3		
None	7.2 ± 1.0	7.3 ± 1.2	7.4 ± 1.3
Endothelin-1 (0.1 nM)	11.0 ± 2.1	11.2 ± 1.9	10.9 ± 1.8
NMB (10 nM)	21.0 ± 1.8	14.3 ± 4.2[a]	7.5 ± 1.0[b]

[a] Significantly different, $p < 0.05$, compared with value without SS octapeptide analogue added.
[b] $p < 0.01$.

tors, SS receptors, and μ-opioid receptors in membranes from the same species, the ability of each of these peptides to inhibit binding of ^{125}I-[Tyr^{11}]SS-14 or ^{125}I-[Tyr^4]Bn to cell membranes from rat pancreatic acinar cell tumor AR42J cells, binding of ^{125}I-Bolton-Hunter-NMB to NMB-R on rat olfactory bulb membranes, and binding of [^{3}H]DAGO to rat forebrain membranes was determined (Table 4). Neither NMB nor GRP at concentrations up to 10 μM inhibited binding of ^{125}I-[Tyr^{11}]SS-14 to SS receptors on AR42J cells or μ-opioid receptors on rat forebrain membranes, and neither SS-14 nor SS-28 at concentrations up to 10 μM inhibited binding to GRP receptors on AR42J cell membranes, NMB-R on rat olfactory bulb membranes, or μ-opioid receptors on rat forebrain membranes (Table 4). There was no correlation between the affinities of the various SS octapeptide analogues for NMB-R on rat olfactory bulb membranes and their affinities for SS receptors on AR42J cells ($r = 0.1$, $p > 0.8$), their affinities for GRP receptors on rat pancreatic membranes ($r = 0.01$, $p > 0.5$), or their affinities for μ-opioid receptors on membranes from rat forebrain ($r = 0.1$, $p > 0.7$) (Table 4). For example, the cyclo-SS analogues cyclo-SS-octa (**1**, Table 1), [Nal^6,Thr^8]-cyclo-SS-octa (**2**, Table 1), [D-Phe^1]-cyclo-SS-octa (**4**, Table 1), [D-Cys^7]-cyclo-SS-octa (**11**, Table 1), and [Phe^6]-cyclo-SS-octa (**13**, Table 1) varied <5-fold in potency for NMB-R yet varied 800-fold in affinity for SS receptors and >5000-fold in affinity for μ-opioid receptors (Table 4). Whereas most of the SS octapeptide analogues had significantly higher affinity for SS receptors than for NMB-R, one analogue, [His^5]-cyclo-SS-octa, had a 3-fold higher affinity for NMB-R. The most potent NMB-R antagonist, cyclo-SS-octa (**1**, Table 4), had a 10-fold greater affinity for NMB-R than μ-opioid receptors, and one analogue (**11**, Table 4) had >50-fold higher affinity. These data demonstrate that the structural requirements of cyclo-SS octapeptides for high affinity NMB-R occupation differ markedly from those for high affinity SS or μ-opioid receptor occupation.

Additional evidence that the various cyclo-SS octapeptide analogues were not altering NMB-R affinity by occupying SS receptors was that no saturable binding of ^{125}I-[Tyr^{11}]SS-14 to glioblastoma C6 cells or the the NMB-R-transfected cells was detected (three experiments). The ^{125}I-[Tyr^{11}]SS-14 used bound to dispersed guinea pig pancreatic acini, which have been shown to possess high affinity SS receptors (38).

Discussion

This study demonstrates for the first time receptor antagonists that have a much higher selectivity for the NMB subtype of Bn receptors than for the GRP subtype of Bn receptors. Previous studies (31) have described five different classses of antagonists for Bn receptors. Three of these classes of antagonists, i.e., des-Met^{14}-Bn or des-Met^{27}-GRP analogues (19, 21, 34), reduced Bn or GRP peptide-bond analogues, and side-chain-restricted analogues, are GRP receptor preferring, with some analogues having a >3000-fold higher affinity for GRP

TABLE 4

Comparison of the affinity of NMB, GRP, SS-14, SS-28, and various SS octapeptide analogues for NMB, GRP, SS, and μ-opioid receptors on plasma membranes from AR42J cells, rat olfactory bulb, or rat forebrain, respectively

Membranes prepared from rat olfactory bulb, rat forebrain, or AR42J cells as described in Experimental Procedures were incubated with the indicated ligands, as described in Experimental Procedures. Affinities were calculated by the method of Cheng and Prusoff (36). Results are means ± 1 SE from at least three experiments.

Analogue	Peptide	K_i			
		AR42J cell membranes		Rat olfactory bulb, ^{125}I-NMB	Rat forebrain, [^{3}H]DAGO
		^{125}I-[Tyr^{11}]SS-14	^{125}I-[Tyr^4]Bn		
		nM			
	NMB	>10,000	19 ± 1	1.1 ± 0.2	>10,000
	GRP	>10,000	1.8 ± 0.1	297 ± 15	>10,000
	SS-14	0.13 ± 0.01	>50,000	>40,000	>10,000
	SS-28	0.40 ± 0.20	>10,000	>10,000	>10,000
1	Cyclo-SS-octa	0.80 ± 0.50	2,870 ± 520	43 ± 9	430 ± 130
2	[Nal^6,Thr^8]-cyclo-SS-octa	0.50 ± 0.10	950 ± 70	85 ± 20	1.9 ± 0.7
4	[D-Phe^1]-cyclo-SS-octa	0.24 ± 0.13	2,000 ± 150	245 ± 130	650 ± 210
5	[Thr^8]-cyclo-SS-octa	0.29 ± 0.03	3,900 ± 1,200	800 ± 200	1.5 ± 0.6
7	[D-Phe^1,Thr^6]-cyclo-SS-octa	0.86 ± 0.23	750 ± 10	740 ± 160	940 ± 320
8	[D-Nal^6]-cyclo-SS-octa	3.9 ± 0.1	4,100 ± 750	1,480 ± 500	206 ± 75
9	[Nal^1,D-Nal^6]-cyclo-SS-octa	2.8 ± 0.7	1,540 ± 180	590 ± 170	1,245 ± 86
10	[D-Cys^2]-cyclo-SS-octa	91 ± 23	1,500 ± 160	920 ± 350	1,910 ± 450
11	[D-Cys^7]-cyclo-SS-octa	48 ± 3	7,620 ± 1,260	230 ± 70	>10,000
12	[D-Trp^1]-cyclo-SS-octa	3.1 ± 1.6	2,100 ± 150	1,180 ± 520	>10,000
13	[Phe^6]-cyclo-SS-octa	194 ± 27	2,150 ± 200	270 ± 90	2,064 ± 424
14	[His^5]-cyclo-SS-octa	1,870 ± 30	3,100 ± 700	470 ± 240	433 ± 90
17	[D-Nal^4]-cyclo-SS-octa	570 ± 180	3,710 ± 200	850 ± 300	5,972 ± 1,668

receptors than NMB-R. Two classes of antagonists, i.e., D-amino acid-substituted SP or SP-4–11 analogues (19, 21) and D-Phe[12]-substituted Bn analogues (19, 21), had 2–3-fold higher affinity for NMB-R than GRP receptors; however, their utility was limited by their low affinity for NMB-R (3–10 μM) and the limited solubility of some [D-Phe[12]]-Bn analogues. Furthermore, the possible usefulness of the D-amino acid-substituted SP or SP-4–11 analogues is limited by their lack of specificity (21). Studies demonstrated that the various D-amino acid-substituted SP analogues were even more potent as SP receptor antagonists than as NMB-R or GRP receptor antagonists. Some SP analogues also inhibited CCK and vasopressin-stimulated activity (39), and in a recent study (40) some such SP analogues interacted directly with G_i or G_o to block agonist stimulation at muscarinic cholinergic M_2 receptors or with G_s to block stimulation by agonist at β-adrenergic receptors in reconstituted phospholipid vesicles.

A number of results in the present study support the conclusion that cyclo-SS octapeptide analogues such as cyclo-SS-octa (1, Tables 1 and 2) are functioning as receptor antagonists with higher affinity for NMB-R than for GRP receptors. First, cyclo-SS-octa had no agonist activity at NMB-R or GRP receptors at concentrations up to 10 μM. Second, cyclo-SS-octa inhibited NMB-stimulated increases in [^{3}H]IP in glioblastoma C6 cells, which have been shown to possess NMB-R (37). Furthermore, these cells also possess endothelin$_A$ receptors, which activate phospholipase C, and cyclo-SS-octa at a concentration that completely inhibited NMB-stimulated increases in [^{3}H]IP had no effect on endothelin-1-stimulated increases in [^{3}H]IP. Third, the NMB stimulation of [^{3}H]IP in C6 glioblastoma cells was inhibited in a competitive fashion by cyclo-SS-octa. Specifically, increasing concentrations of cyclo-SS-octa caused a parallel rightward shift in the NMB-stimulated dose-response curve, with no change in maximal stimulation, and the extent of the shift was dependent on the NMB and cyclo-SS-octa concentrations. Furthermore, plotting the data in the form of Schild demonstrated an affinity of 231 nM and a slope of the regression equation not significantly different from unity, demonstrating competitive interaction characteristic of a receptor antagonist. Fourth, cyclo-SS-octa inhibited ^{125}I-[D-Tyr^0]NMB binding to NMB-R on both C6 glioblastoma cells and cells transfected with NMB-R from rat esophagus. Furthermore, cyclo-SS-octa inhibited NMB binding by decreasing NMB-R affinity without altering the total number of NMB-R, which is characteristic of a receptor antagonist. Fifth, the various cyclo-SS octapeptide analogues inhibited NMB-stimulated increases in [^{3}H]IP and binding with the same relative affinities, demonstrating that inhibition of NMB interaction with the NMB-R could account for their abilities to inhibit the action of NMB. Lastly, for both inhibition of binding to NMB-R and GRP receptors and inhibition of the ability of NMB or GRP to alter cell activity, cyclo-SS-octa had >100-fold higher affinity for NMB-R than for GRP receptors, demonstrating its selectivity for NMB-R over GRP receptors.

In previous studies SS-14 was reported to interact weakly with opiate receptors (27, 28). Subsequently, various D-amino acid- and conformationally restricted amino acid-substituted cyclo-SS octapeptide analogues were described (28–30) that functioned as potent μ-opioid receptor antagonists with low affinity for κ- or δ-opioid receptors. Furthermore, a number of amino acid-substituted cyclo-SS octapeptide analogues (41) functioned as potent SS receptor agonists. In the present study, the fact that there was no correlation between the relative affinities of the various cyclo-SS octapeptide analogues for NMB-R and for μ-opioid or SS receptors, as well as the inability to demonstrate SS receptors on either C6 glioblastoma cells or 3T3 fibroblasts transfected with NMB-R, supports the conclusion that these cyclo-SS octapeptide analogues were not inhibiting the action of NMB by interacting with SS or μ-opioid receptors. This conclusion is further supported by the inability of SS-14 or SS-28 to alter NMB-R interaction at concentrations >20,000-fold higher than those that inhibit binding to SS receptors on AR42J cells. The fact that there was no correlation between the affinities of the various cyclo-SS octapeptide analogues for NMB-R and for μ-opioid or SS receptors demonstrates that there are different SS peptide structural requirements for occupation of these three receptors. Therefore, in the present study, whereas the most potent NMB-R antagonist, cyclo-SS-octa, had only a 10-fold higher affinity for NMB-R than for μ-opioid receptors and a 40-fold higher affinity for SS receptors than for NMB-R, it is likely that in the future specific high affinity NMB-R antagonists will be developed that will have high selectivity for NMB-R over SS or μ-opioid receptors. This conclusion is further supported by studies showing that the affinities of cyclo-SS octapeptide analogues for μ-opioid and SS receptors differed, and subsequently cyclo-SS octapeptide analogues were developed that had a >10,000-fold selectivity for μ-opioid over SS receptors (29).

The present study provides some preliminary insights into the importance of the various substitutions in cyclo-SS-octa in determining NMB-R affinity, as well as the affinity for μ-opioid or SS receptors (Fig. 5). The hydrophobicity of the group in position 1 appears to be more important for NMB-R affinity than for SS or μ-opioid receptor affinity, because changing D-Nal[1] to the less hydrophobic group D-Phe[1] caused a >5-fold decrease in affinity for the NMB-R but caused either no change in affinity (SS receptor) or a 1.5-fold decrease in affinity (μ-opioid receptor) (compare 1 and 4, Tables 1 and 4). However, the type of ring substitution in position 1 seems particularly important for μ-opioid affinity, because substitution of D-Trp[1]

Structure	Position	Alteration	Affinity for receptor for: NMB	Mu Opioid	NMB
D-Nal	1	↓ hydrophobicity	↓ (5x)	NC	NC
		Ring type	↓↓ (20x)	↓↓↓↓ (inactive)	↓ (6x)
		Stereochemistry	↓↓ (12x)	↓ (3x)	NC
Cys	2	Stereochemistry	↓↓ (20x)	↓ (5x)	↓↓↓ (90x)
Thr	3	–			
D-Trp	4	Ring size/ hydrophobicity	↓↓ (20x)	↓(15x)	↓↓↓(600x)
Lys	5	Need for Lys	↓ (10x)	NC	↓↓↓↓(1000x)
Val	6	–			
Cys	7	Stereochemistry	↓ (5x)	↓↓↓↓ (inactive)	↓↓(50x)
Nal	8	Stereochemistry	↓↓ (30x)	↓ (2x)	↓ (5x)
NH_2		Thr[8] for Nal[8]	↓↓ (20x)	NC	↓↓↓(200x)

Fig. 5. Summary of effects of various amino acid substitutions in cyclo-SS-octa on affinities for NMB, μ-opioid, and SS receptors. Results are from data in Table 4. *Direction of the arrows*, direction of change in affinity produced by the substitution (↑, increased affinity; ↓, decreased affinity). The fold change in affinity is indicated by the number in parentheses and by the number of arrows (↓, 1–5-fold change; ↓↓, 6–50-fold change; ↓↓↓, 60–999-fold change; ↓↓↓↓, >1000-fold change). *NC*, no change.

for D-Nal[1] caused a complete loss of μ-opioid binding activity (compare **1** and **12**, Tables 1 and 4). The stereochemistry of the substitution in position 8 was much more important for determining the affinity for the NMB-R than that for the μ-opioid or SS receptor (compare **1** and **8**, Tables 1 and 4), whereas the stereochemistry of the amino acid in position 1 had a greater effect on μ-opioid receptor affinity than on NMB-R or SS receptor affinity (compare **8** and **9**, Tables 1 and 4). The stereochemistry of the cysteine in position 7 was much more important for determining μ-opioid or SS receptor affinity than NMB-R affinity (compare **1** and **11**, Tables 1 and 4), whereas the stereochemistry of Cys[2] was more important in determining SS receptor and NMB-R affinity than μ-opioid receptor affinity (compare **1** and **10**, Tables 1 and 4). The substitution of Thr[8] for Nal[8] in cyclo-SS-octa caused a >200-fold increase in affinity for μ-opioid receptors, with a minimal effect on SS affinity; however, this decreased NMB-R affinity by >20-fold (compare **1** and **5**, Tables 1 and 4). In contrast, the hydrophobicity and ring size of the amino group in position 4 were much more important in determining SS receptor affinity, compared with either NMB-R or μ-opioid receptor affinity (compare **1** and **17**, Tables 1 and 4). Similarly, the need for Lys[5] was much greater in determining SS receptor affinity than either NMB-R or μ-opioid receptor affinity (compare **1** and **14**, Tables 1 and 4). These results suggest that alterations in either the sterochemistry or the nature of the substitutions in positions 4, 7, and 8, as well as position 5, of cyclo-SS-octa are particularly important for retaining high affinity NMB-R interaction and decreasing SS or μ-opioid receptor interaction and, therefore, modifications in these positions in the future will likely lead to a much more selective NMB-R antagonist (Fig. 5). It is tempting to speculate that the obviously important aromatic groups present in these SS analogues induce NMB-R binding by mimicking the three aromatic side chains present in the NMB sequence (Gly-Asn-Leu-Trp-Ala-Thr-Gly-His-Phe-Met-NH_2). How this is accomplished may become more obvious as additional structure-activity data are gathered. Also, because the SS octapeptide analogues are relatively restricted conformationally, compared with the linear sequence, and in several cases have been shown by two-dimensional ^1H NMR studies (42) to adopt hydrogen-bonded folded structures in solution, it is hoped that they will provide a template for deducing the receptor-bound conformation(s) of NMB and its analogues.

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