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Gastrin-Releasing Peptide Receptor in Rat Brain Membranes: Specific Binding and Stimulation of Phosphoinositide Breakdown

Elizabeth B. Hollingsworth

Pharmacology Division, The Wellcome Research Laboratories, Research Triangle Park, North Carolina 27709

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SUMMARY

The binding of gastrin-releasing peptide (GRP) to rat brain membranes was characterized. GRP binds specifically to a high affinity site in rat brain membranes, with a K_d equal to 2 nM and B_{max} equal to 5 pmol/g wet weight of tissue. The specific binding is saturable, reversible, and dependent on tissue concentration, time of incubation, and the pH of the buffer. Hippocampus, cortex, and striatum contained the highest concentration of high affinity binding sites and the thalamus the lowest. The affinities of GRP, bombesin, and their analogues for the GRP receptor were determined. GRP(14–27) and [Tyr^4]bombesin had the greatest affinity, whereas GRP(1–16), which lacks the COOH terminal region, had no affinity for the receptor. GRP, bombesin, and analogues stimulate the breakdown of phosphatidylinositol in rat brain hippocampal minces and potencies correspond to their affinities for the GRP receptor.

The brain-gut peptide GRP was first detected in mammalian tissue in 1979 (1). GRP is a peptide of 27 amino acids that displays strong homology at its COOH-terminal region to another brain-gut peptide, bombesin (2). There are a number of different bombesin-like peptides, which have been localized in mammalian gastrointestinal tract (1, 3) lung (4–6), and brain (3, 7–10). These peptides have potent pharmacological effects in the central nervous system as well as in the peripheral nervous system (11–29). For example, bombesin and GRP both inhibit gastric acid secretion when they are administered into the lateral, third, or fourth ventricle (30). Injections of bombesin into the periaqueductal gray matter induces an apparent analgesia, which is not antagonized by naloxone (23). Bombesin also modulates the dopamine system (17) and chronic bombesin treatment alters the affinity of [^{3}H]spiperone for the dopamine-2 receptor (18).

Recently Moody *et al.* (31) reported on the specific binding of bombesin in rat brain. However, bombesin is not found in mammalian tissues (32, 33). Because GRP is probably the more relevant peptide in mammals, the specific binding of GRP has been characterized in rat brain in this study.

Experimental Procedures

Materials. ^{125}I-GRP was obtained from Amersham (Arlington Heights, IL), [^{3}H]inositol from DuPont (Boston, MA), GRP and bombesin from Sigma Chemical Co. (St. Louis, MO), and GRP(14–27), GRP(20–27), GRP(1–16), and [Tyr^4]bombesin from Peninsula Laboratories (Belmont, CA). Other chemicals used came from standard sources. Peptide stocks (100 μM) were in water and were stored at −80° for no longer than 2 weeks. Poly-Prep (2 ml) Econo-Columns were obtained from Bio-Rad (Richmond, CA).

Preparation of membranes. Adult male Sprague-Dawley rats (175–200 g) were decapitated and the brains were dissected. The hippocampus was removed and used for the assay unless noted otherwise. Hippocampal tissue was homogenized in 5 volumes of 0.32 M sucrose with a Teflon pestle homogenizer. This homogenate was centrifuged at 1200 × *g* for 10 min. The supernatant was then centrifuged for 10 min at 40,000 × *g*. The pellet was dispersed with a sonicator in 5 volumes of 50 mM Tris (pH 7.4 at 0°) for 1 min. This homogenate was centrifuged for 10 min at 40,000 × *g*. The pellet was suspended in 50 mM Tris plus 1% BSA to give a tissue concentration corresponding to 10 mg wet weight of tissue/0.2 ml.

Binding assay. Routinely, 200 μl of fresh homogenate (10 mg wet weight, ≃ 0.115 mg of protein) were incubated with 0.15 nM ^{125}I-GRP at 0° for 30 min, in the presence or absence of competitor (1 μM GRP or bombesin, as described in figure legends) to determine nonspecific binding. The assay buffer composition was 50 mM Tris that contained 1% BSA and bacitracin (2 μg/ml), pH 7.4 at 0°. The assay volume was 0.5 ml and each assay was performed in triplicate in polystyrene tubes (Sarstedt).

Membrane-bound ^{125}I-GRP was separated from free peptide by filtration under reduced pressure through GF/B filters (Whatman) that were presoaked at least 1 hr with 1% BSA and 1% polyethyleneimine in 50 mM Tris. Such treatment markedly reduces filter binding. The filters were rinsed promptly twice with 3 ml of ice-cold 50 mM Tris, pH 7.4 at 0°, that contained 0.1% BSA. This filtration was carried out using a single-manifold filtration apparatus with silicated glass. The filters were assayed for radioactivity in a Packard γ counter. Analysis of the saturation experiments was carried out using the LIGAND program developed by Rodbard and Munson (34).

Phosphatidylinositol breakdown assay. The assay is essentially

ABBREVIATIONS: GRP, gastrin-releasing peptide; BSA, bovine serum albumin.

as described by Gusovsky and Daly (35). Briefly, adult male Sprague-Dawley rats (175–200 g) were decapitated and the brain region was dissected out. Brain tissue was minced with a razor blade into a homogeneously sized preparation. The tissue was then incubated at 37° for 30 min in 20 ml of a Krebs-Henseleit buffer. After 15 min, the buffer was replaced with fresh buffer. The buffer was decanted and the tissue was evenly distributed, with a 50-μl repeator pipette, into plastic vials. The end (2 mm) of the pipette was removed to aid tissue distribution. [^{3}H]Inositol (0.35 nmol) was added to each vial (total volume of 400 μl). The plastic vials were exposed to O_2/CO_2 (95%:5%) for 5 sec, capped, and incubated (37°) for 60 min. Tissue was then preincubated with 10 mM LiCl (10 min) before the addition of the peptide and again vials were incubated for 60 min under the O_2/CO_2 conditions described above. The assay mixture was transferred to Eppendorf centrifuge tubes and centrifuged (13,000 × *g*) for 30 sec. The pellet was washed once with buffer and centrifuged and the buffer was removed by aspiration. Next, the washed pellet was digested with 1 ml of 6% trichloroacetic acid and recentrifuged. The supernatant was added to Dowex (AG1-X8, 100-200 mesh, formate form, 0.5 ml) columns. Samples were analyzed essentially as described by Berridge *et al.* (36). All columns were washed five times with 3 ml of water to remove free [^{3}H]inositol. [^{3}H]Inositol monophosphate retained on the columns was eluted with 2 ml of ammonium formate (200 mM) in formic acid (100 mM). Verification of this elution scheme is reported by Hollingsworth and Daly (37). Aquasol 2 (New England Nuclear, Boston, MA) was added and the mixture was analyzed for radioactivity in a scintillation counter. Total membrane [^{3}H]inositol incorporation was determined by the addition of 500 μl of a lipid-extracting solution (1 M KCl, 10 mM inositol in 1:1 ratio with methanol) plus 500 μl of $CHCl_3$ to the trichloroacetic acid precipitate (see above). The tubes were capped and agitated vigorously for 5 min and then centrifuged for 30 sec. The chloroform phase was removed and counted in a scintillation counter in the presence of Aquasol-2. Analysis of the data was carried out by dividing the total amount of [^{3}H]inositol monophosphate recovered from the column by the total amount of radioactivity in the lipids. These calculations give an estimation of the quantity of phosphatidylinositol broken down to inositol monophosphate due to receptor stimulation (35).

Results

Binding of ^{125}I-GRP to membranes. Fig. 1 illustrates that specific binding can be achieved with ^{125}I-GRP using rat brain membranes. Specific binding was determined by subtracting total ^{125}I-GRP binding from nonspecific binding (binding of ^{125}I-GRP in the presence of 1 μM GRP. Specific binding increased in a linear fashion up to approximately 20 mg wet weight of tissue (200–400 μg of protein) and then started to plateau.

Equilibrium for specific binding was reached by 30 min (Fig. 2). Nonspecific binding (1 μM GRP) reaches equilibrium by 5 min, whereas total binding reaches equilibrium by 30 min. It should be noted that binding was performed at 0° because the specific binding was poor when the experiment was run at 37°. The cold temperatures perhaps slow the proteolytic degradation or denaturation of the peptides or the receptor. Using thin layer chromatography, it was verified that the ligand was not breaking down under the assay conditions stated, i.e., the ligand comigrated with the GRP after exposure to the assay conditions (data not shown).

Specific binding was greatest between pH 7.0 and 7.4 and the addition of NaCl or KCl (12 mM and higher) to the buffer decreased binding (data not shown). As shown in Fig. 3, specific binding was reversible. Dissociation of specific binding was achieved in the presence of GRP (1 μM).

Multiple saturation experiments were carried out using concentrations of ^{125}I-GRP up to 15 nM with bombesin (1 μM) to determine nonspecific binding. Specific binding reached a maximum with 10 nM concentrations of ^{125}I-GRP. The saturation curves were analyzed by Scatchard analysis using the LIGAND program of Rodbard and Munson (34). Three combined analyses revealed one binding site with a K_d equal to 2.0 ± 9 mM and a B_{max} equal to 5.0 ± 0.8 pmol/g of wet weight of tissue (435 fmol/mg of protein). The Hill coefficient was close to unity. Another set of saturation experiments were performed using GRP (1 μM) to determine nonspecific binding instead of bombesin. Saturation was also seen in these experiments in the 10 nM range (Fig. 4). Scatchard analysis revealed a K_d equal to 10 nM and a B_{max} equal to 25 pmol/g wet weight of tissue. A second experiment produced a K_d equal to 4 nM and a B_{max} equal to 12 pmol/g wet weight (data not shown). These results indicate that GRP may bind to more sites than bombesin,

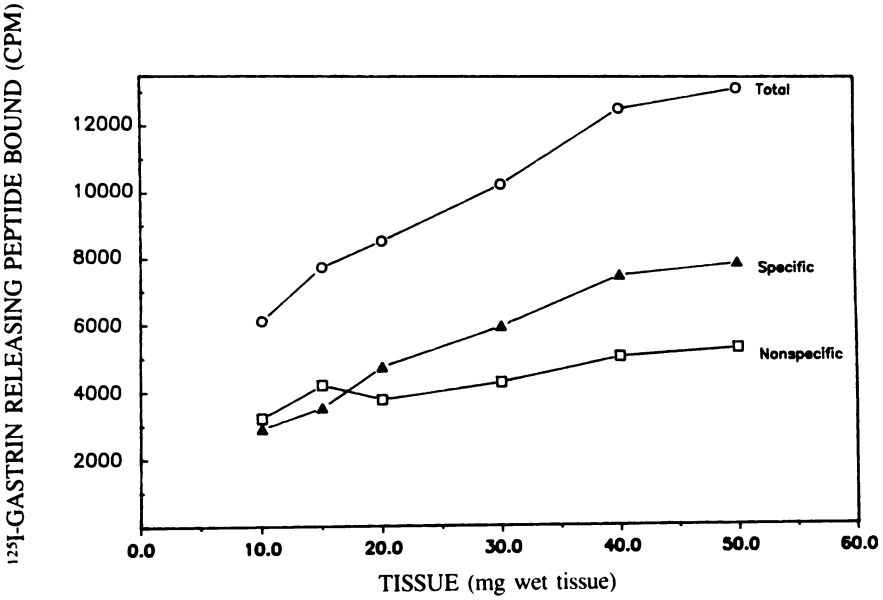

Fig. 1. Effect of tissue concentration on ^{125}I-GRP specific binding in rat brain membranes. The binding of ^{125}I-GRP (0.15 nM) to rat brain membranes alone (total) (○) and in the presence of GRP (1 μM) (nonspecific) (□) was determined as described in Experimental Procedures. Specific binding (▲) is calculated as total minus nonspecific. The results are from a typical experiment repeated two more times. Filter blanks for this experiment would have been approximately 800 cpm.

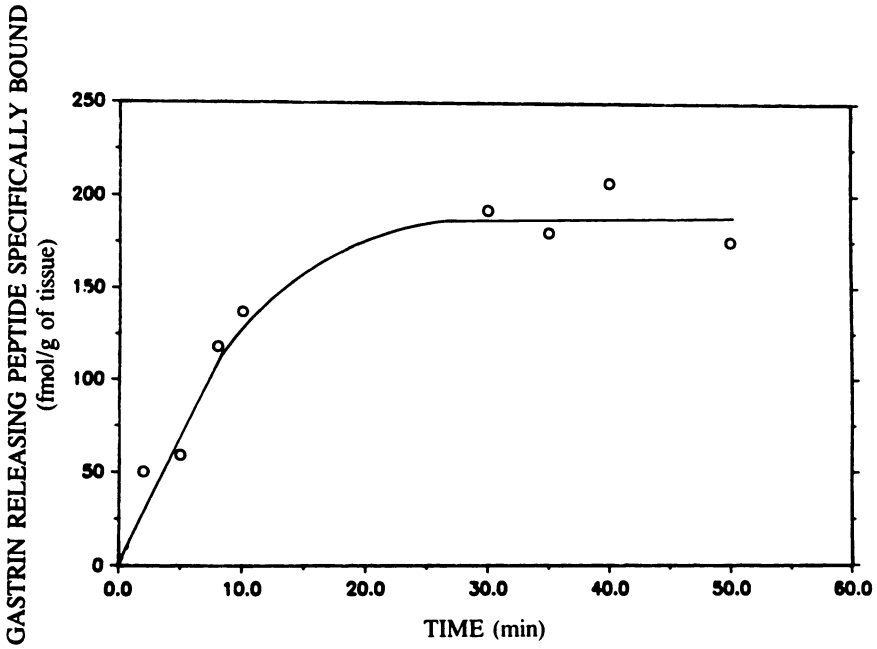

Fig. 2. Effect of time on specific binding of ^{125}I-GRP to rat brain membranes. ^{125}I-GRP (0.15 nM) was incubated in the presence of GRP (1 μM) to determine nonspecific binding. The results are from a typical experiment repeated two more times.

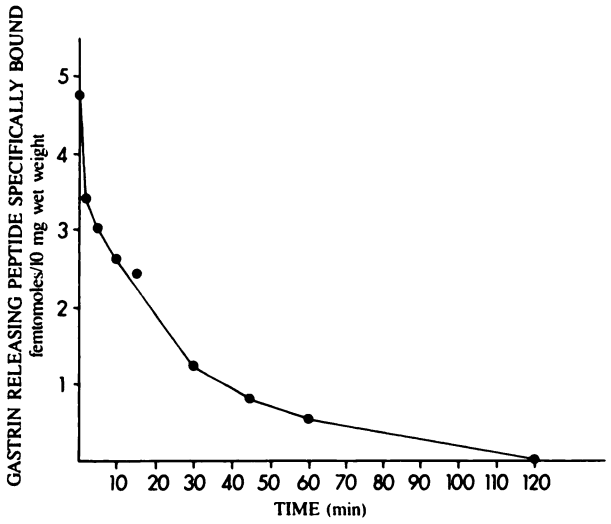

Fig. 3. Dissociation of the specific binding of ^{125}I-GRP to rat brain membranes. ^{125}I-GRP (0.15 nM) was incubated using the assay conditions stated in Experimental Procedures for 30 min at 0° to achieve equilibrium. GRP (1 μM, 5 μl) was added, to initiate dissociation, for the times indicated to determine the time needed to reverse total binding. The results are from a typical experiment repeated two more times.

because the B_{max} appears to be larger when nonspecific binding is determined with GRP instead of bombesin. More data would have to be collected to determine whether this is true or whether the difference is due to assay variability.

Regional binding studies (Table 1) revealed the highest density of ^{125}I-GRP binding sites in the cortex, hippocampus, and striatum. The lowest densities were observed in the midbrain, medulla-pons, and thalamus.

Inhibition of ^{125}I-GRP specific binding with GRP and analogues. Analogues of GRP and bombesin were tested for their ability to inhibit ^{125}I-GRP (0.15 nM) binding to rat membranes. IC_{50} values were determined as the concentrations that inhibited specific binding by 50%. The 27-amino acid peptide GRP and GRP(14–27) had similar IC_{50} values, equal to 2.2 and 1.5 nM, respectively, whereas GRP(20–27) had an IC_{50} value equal to 10 nM (Table 2). These were not statistically different. GRP(1–16) showed no affinity towards the receptor. Bombesin and [Tyr^4]bombesin were the only two peptides to give statistically different IC_{50} values, which were equal to 4.3 and 0.9 nM, respectively. The bombesin peptides, therefore, have an affinity similar to the GRP peptides for the GRP receptor.

Effect of GRP, bombesin, and analogues on phosphatidylinositol breakdown. The effect of these peptides on the breakdown of phosphalidylinositol was explored in hippocampal slices. A time-course study (Table 3) revealed that a maximal stimulation by bombesin was reached by 60 min. Beyond 30 min, basal values increased at the same rate as those detected in the presence of the peptide; therefore, percent control values no longer increase. An EC_{50} value for bombesin of approximately 2 nM was determined from the concentration-response curve (Fig. 5). At concentrations of 10 nM, the effect of bombesin on phosphatidylinositol turnover reached a maximum and remained constant up to concentrations equal to 1 μM. The ability of bombesin (1 and 10 μM) to stimulate the breakdown of phosphatidylinositol (Table 1) was determined in different brain regions. The greatest stimulation was in the hippocampus and striatum. No significant stimulation was seen in the medulla-pons or midbrain. The potency of the relevant peptides in stimulating the breakdown of phosphatidylinositol in hippocampal tissue is shown in Table 2. GRP, bombesin, and their analogues have EC_{50} values in the nanomolar range, none of which are statistically different except for GRP(1–16), which was inactive.

Discussion

GRP binds to rat brain membranes. The binding is specific and saturable (5 pmol/g of wet tissue). Saturation experiments produced Hill coefficients close to unity, indicating one class of noninteracting sites. Characteristics of the high affinity GRP site are similar to the K_d and B_{max} values (5.5 nM and 4 pmol/mg of tissue, respectively) for the specific binding of ^{125}I-bombesin in rat brain membranes (31). Regional distribution studies revealed that the greatest concentration of GRP binding

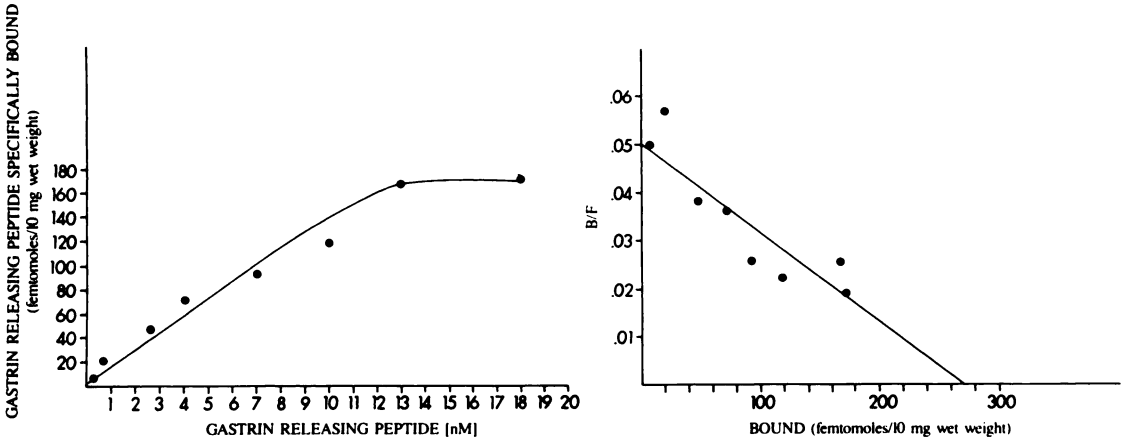

Fig. 4. Specific binding of GRP to rat brain membranes as a function of GRP concentration. The binding was measured after membranes were incubated (10 mg wet weight) for 30 min at 0° with increasing concentrations of ^{125}I-GRP in the presence or absence of GRP (1 μM) to determine nonspecific binding. Each *point* is the mean of triplicate determinations in a single experiment. Similar results were obtained in a separate experiment. Data were analyzed according to Scatchard analysis using the LIGAND program of Munson and Rodbard (34) to obtain K_d and B_{max} values.

TABLE 1

Regional distribution of specific ^{125}I-GRP binding and bombesin-induced phosphatidylinositol breakdown in rat brain minces

Saturating concentrations of ^{125}I-GRP (15 nM) were used to determine the distribution of GRP binding sites in different brain regions. Nonspecific binding was determined with GRP(14–27) (10^{-5} M). The breakdown of phosphatidylinositol was measured after 60-min stimulation with maximally effective concentrations of bombesin (1 and 10 μM). The experimental methods and analysis are described in Experimental Procedures. Results are expressed as mean ± standard error of three or four experiments, performed in triplicate or quadruplicate.

Brain Region	^{125}I-GRP Specifically Bound	Phosphatidylinositol Breakdown
	pmol/mg of protein	*% of control*
Hippocampus	0.701 ± 0.19	146 ± 2
Cortex	0.768 ± 0.12	117 ± 5
Striatum	0.970 ± 0.12	139 ± 7
Hypothalamus	0.555 ± 0.18	119 ± 23
Cerebellum	0.400 ± 0.05	107 ± 3
Medulla-pons	0.087 ± 0.06	103 ± 6
Midbrain	0.063 ± 0.08	107 ± 5
Thalamus	0	

TABLE 2

Activity at the GRP receptor of GRP, bombesin, and analogues

Binding assays were performed using 0.15 nM ^{125}I-GRP and increasing concentrations of the peptides listed below to determine the concentrations needed to inhibit maximum binding by 50% (IC_{50}). Values are expressed as means ± standard errors of three or four experiments. Data were analyzed using an analysis of variance followed by a group *t* test. Concentration-response curves were performed for each peptide for their ability to stimulate the breakdown of phosphatidylinositol. Assay conditions are described in Experimental Procedures. EC_{50} values were determined from concentration-response curves derived from three or four experiments.

Peptide	Receptor Binding, IC_{50}	Stimulation of Phosphatidylinositol Breakdown, EC_{50}
	nM	
GFP	2.2 ± 1.3	3.0 ± 1.0
GRP(14–27)	1.5 ± 0.4	3.7 ± 1.3
GRP(20–27)	10.0 ± 3.9	
GRP(1–16)	No effect (10^{-6} M)	Not active
Bombesin	4.3 ± 1.1[a]	1.3 ± 0.3
[Tyr^4]-Bombesin	0.9 ± 0.2	3.5 ± 1.4

[a] Statistically different from [Tyr^4]-Bombesin.

TABLE 3

Time course for bombesin-induced phosphatidylinositol breakdown

The breakdown of phosphatidylinositol in rat brain hippocampus minces was determined after stimulation for various times with bombesin (1 μM). The experimental methods are described in Experimental Procedures. The results are the means of an experiment run in triplicate.

Incubation Time	Inositol Monophosphates Eluted	
	Control	Bombesin (1 μM)
min	*dpm*	
5	1074	1307
8	1212	1416
15	1243	1497
30	1314	1763
45	1331	1861
60	1440	1937

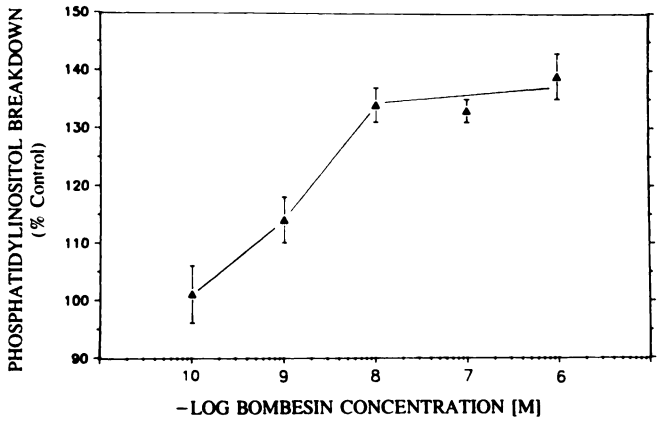

Fig. 5. Effect of bombesin on the breakdown of phosphatidylinositol in rat brain hippocampal minces. The breakdown of phosphatidylinositol was determined after 60-min stimulation with bombesin at various concentrations. The experimental methods and analysis are described in Experimental Procedures. Results are expressed as percentage of control values, giving the mean ± standard error of three experiments run in quadruplicate.

occurs in the cortex, striatum, and hippocampus. These results parallel what has been found by Moody *et al.* (31) for density of ^{125}I-bombesin binding sites. One exception is that specific thalamic ^{125}I-bombesin binding was detected. The parallels seen in K_d and B_{max} values and regional distributions between ^{125}I-

bombesin (31) and ^{125}I-GRP specific binding suggest that these peptides are binding to the same population of sites.

In the hippocampus, concentrations of bombesin and GRP are quite low (9), whereas high concentrations of the GRP and bombesin (9) binding sites are detected. Herkenham (38) indicates that it is not unusual in the field of peptide research for there to be disparity between peptide content and the corresponding receptor density in various brain regions. He proposes that peptides can affect sites not only in close proximity but also distally to where they are released.

The regional distribution binding and second messenger studies reveal that the areas with the greatest amount of ^{125}I-GRP specific binding also show the most robust stimulation of phosphatidylinositol breakdown by bombesin. Areas such as the midbrain and medulla-pons that show little significant binding also showed no significant production of the second messenger by bombesin. Receptor distribution parallel with second messenger production supports the postulation of a physiological relevance to this binding site. This effect of bombesin on phosphatidylinositol turnover is also seen in Swiss 3T3 cells (39, 40) but this is the first published report on the effect of bombesin and GRP on this second messenger system in brain tissue.

Analogous to the similar IC_{50} values for the binding results (Table 2), GRP, bombesin, and relevant analogues produce roughly equivalent EC_{50} values for the stimulation of the breakdown of phosphatidylinositol. Although two of the peptides (bombesin and [Tyr^4]bombesin) have significantly different IC_{50} values for binding, the EC_{50} values determined for stimulation of phosphatidylinositol are not statistically different. Because the second messenger assay is run at 37°, it is possible the peptides are degraded. Degradation at different rates during the 60-min incubation could explain the discrepancy between affinities for the receptor and potencies at the receptor for stimulation of phosphatidylinositol breakdown.

Peptide affinity can be associated with agonist or antagonistic activity, however, and lack of affinity can be correlated with no efficacy at stimulating the breakdown of phosphatidylinositol. The rough parallel seen between the affinity of the peptides for the ^{125}I-GRP binding site and their potency in breaking down phosphatidylinositol again indicates that this binding site has physiological relevance. GRP(1–16), which lacks the COOH-terminal region, was inactive at stimulating this second messenger system, which correlates with its lack of affinity at the GRP receptor. Other peptides such as neurotensin (41) also do not require the COOH terminal region for affinity or efficacy at its receptor.

This study has fulfilled many of the criteria for verifying that a binding site actually represents the receptor for a drug or a neurotransmitter. ^{125}I-GRP specific binding is saturable and reversible, shows brain regional distribution, and is pH, temperature, ion, and tissue concentration dependent. Binding has also been associated with a response, i.e., stimulation of phosphatidylinositol breakdown. Because GRP appears to be the relevant mammalian peptide, future neurochemical and behavioral work focusing on its actions, such as its modulation of phosphatidylinositol breakdown, will be of interest.

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Send reprint requests to: Elizabeth Hollingsworth, Pharmacology, The Wellcome Research Laboratories, 3030 Cornwallis Rd., Research Triangle Park, NC 27709.