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Bombesin, neuromedin B and neuromedin C interact with a common rat pancreatic phosphoinositide-coupled receptor, but are differentially regulated by guanine nucleotides

M. Chandra SEKAR,* Naomi UEMURA,* David H. COY,† Basil I. HIRSCHOWITZ* and Kenneth E. J. DICKINSON*‡

*Division of Gastroenterology, University of Alabama at Birmingham, Birmingham, AL 35294, U.S.A., and †Peptide Research Labs, Tulane University School of Medicine, New Orleans, LA 70112, U.S.A.

Bombesin (BB), neuromedin C (NMC) and neuromedin B (NMB) stimulated amylase secretion to similar maximum levels, with EC_{50} values (concentrations causing 50% of maximum effect) of 0.2, 0.3 and 2 nM respectively. Treatment of pancreatic acini with BB or NMB (10 nM) for 30 min resulted in cross-desensitization of secretory responses to subsequent BB and NMB, but not to acetylcholine, which suggests that NMB and BB activate the same receptor. BB, NMC and NMB stimulated production of similar maximum amounts of inositol mono-, bis- and tris-phosphates, with EC_{50} values of 3, 5 and 141 nM respectively. The bombesin receptor antagonist [Leu^{13}-$\psi(CH_2NH)Leu^{14}$]BB inhibited stimulation of amylase secretion and inositol phosphate formation by BB, NMC and NMB. Binding of ^{125}I-labelled gastrin-releasing peptide (GRP; 200 pM) to rat pancreatic membranes at 22 °C was inhibited with relative potencies and IC_{50} (concn. causing 50% of maximal inhibition; nM) as follows: NMC (0.4) = BB (0.5) > NMB (1.8) = GRP (2.6). IC_{50} values for BB, NMC and NMB inhibition of ^{125}I-GRP binding to intact acini were 5-, 19- and 68-fold higher than their respective values in membranes. The guanine nucleotide analogue guanosine 5′-[$\beta\gamma$-imido]triphosphate (Gpp[NH]p) produced rightward shifts of NMC and NMB competition curves by 3.5- and 16-fold respectively, but had little effect on the BB and GRP curves. Elevation of the temperature to 37 °C or inclusion of NaCl (40 mM) produced quantitatively similar effects to those of Gpp[NH]p. In the presence of both NaCl and Gpp[NH]p the affinities of peptides for membrane receptors were similar to those for intact cells. Modulation of NMB competition curves by Gpp[NH]p was not attenuated by prior treatment of acini with activated pertussis toxin. These results suggest that BB, NMB and NMC stimulate pancreatic secretion by interaction with a common phosphoinositide-linked receptor. Differences in guanine nucleotide regulation suggest that secretagogue-induced receptor–protein interactions may not be identical for NMB and BB.

INTRODUCTION

The tetradecapeptide bombesin (BB) and its mammalian counterpart gastrin-releasing peptide (GRP) are of major interest in view of their activities as secretagogues in mammalian pancreatic acini [1], pituitary cells [2], gastrin G cells [3] and amphibian peptic cells [4]. BB also functions as a growth factor for Swiss 3T3 fibroblasts [5], pancreatic and gastrin G cells [6] and small cell lung cancer cells, where it may stimulate the release of a BB-like peptide [7].

Minamino *et al.* [8] recently purified a decapeptide from pig spinal cord which corresponded to pig GRP-(18–27)-peptide, which they called neuromedin C (NMC). NMC differed from bombesin-(5–14)-peptide in a single amino acid substitution at position 7. These workers also reported the purification and sequence of a decapeptide termed neuromedin B (NMB) which had amino acid substitutions at positions 3, 6 and 9 of NMC [9]. NMB and NMC have been reported to stimulate amylase secretion from the rat pancreas [10]. The affinity of NMB for rat oesophageal BB receptors (K_i 0.3 nM) has been reported to differ from that for the rat pancreas (K_i 156 nM), which suggested the existence of subtypes of BB receptors in these tissues [11]. We have also provided evidence that BB receptors of frog oesophageal peptic cells differ from rat pancreatic receptors in their response to the BB receptor antagonist [Leu^{13}-ψ-(CH_2NH)-Leu^{14}]BB (LL-BB) [12].

The present study has examined the concept that BB and neuromedins stimulate cells through distinct receptors. We have examined the interaction of BB, NMB and NMC with rat pancreatic acini in order to detect differences in functional response, second messenger formation, desensitization and receptor binding characteristics. The results suggest that BB and the neuromedins stimulate a common BB receptor, but that guanine nucleotides regulate secretagogue binding to membranes differently for BB and neuromedins.

MATERIALS AND METHODS

Materials

Purified collagenase (500 units/mg) was purchased from Worthington, Freehold, NJ, U.S.A. Guanosine 5′-[$\beta\gamma$-imido]-triphosphate (Gpp[NH]p; tetralithium salt) was from Boehringer–Mannheim, Indianapolis, IN, U.S.A. The peptides BB, litorin, [Tyr^4]BB, ranatensin, GRP, NMC, NMB and NMB-30 were from Peninsula Labs, Belmont, CA, U.S.A. LL-BB was synthesized by D. H. Coy using previously described techniques [13]. Minimal essential medium amino acids, soybean trypsin inhibitor and BSA were purchased from Sigma Chemical Co., St.

Abbreviations used: BB, bombesin; NMB, neuromedin B; NMC, neuromedin C; GRP, gastrin-releasing peptide, LL-BB, [Leu^{13}-$\psi(CH_2NH)$-Leu^{14}]BB; Gpp[NH]p, guanosine 5′-[$\beta\gamma$-imido]triphosphate; IC_{50}, concn. causing 50% of maximal inhibition; EC_{50}, concn. causing 50% of maximal effect; CCK, cholecystokinin.

‡ To whom correspondence should be sent, at present address: Department of Biochemistry, Bristol Myers–Squibb Pharmaceutical Research Institute, P.O. Box 4000, Princeton, NJ 08543, U.S.A.

Louis, MO, U.S.A. *myo*-[^{3}H]Inositol (20 Ci/mmol) was from New England Nuclear, Boston, MA, U.S.A.; ^{125}I-GRP (2200 Ci/mmol) was from Amersham, Arlington Heights, IL, U.S.A.; and ^{125}I-[Tyr^4]BB was generously provided by New England Nuclear. The standard ^{3}H-labelled inositol phosphates Ins1P_1, Ins4P_1, Ins(1,4,5)P_3, Ins(1,3,4)P_3 and Ins(1,3,4,5)P_4 were purchased from New England Nuclear.

Preparation of rat pancreatic acini and assay of amylase activity

Pancreatic acini and minilobules were prepared by collagenase digestion of pancreatic tissue obtained from Sprague–Dawley rats (250–300 g) as described previously [14]. Isolated acini were suspended in Krebs–Henseleit medium (pH 7.4; O_2/CO_2, 19:1) supplemented with minimal essential medium amino acids, glucose (11 mM), soybean trypsin inhibitor (0.01 %), BSA (0.2 %) and 2.5 mM-Ca^{2+}. Acini were pre-incubated at 37 °C for 30 min, and then portions were transferred into Eppendorf centrifuge tubes (total vol. 1 ml) and incubated with secretagogues for 30 min at 37 °C. Acini were centrifuged and samples of supernatant were assayed for amylase activity using the method of Rick & Stegbauer [15]. The total amylase content of acini before incubation was determined and amylase released/30 min was expressed as a percentage of the total, or normalized to the maximum secretion elicited by bombesin.

Preparation of rat pancreatic membranes

Rat pancreatic acini were prepared and homogenized in 5 ml of buffer A (50 mM-Tris/5 mM-$MgCl_2$, pH 7.4) using a Potter–Elvehjem homogenizer. The homogenate was passed through two layers of muslin and centrifuged at 40000 *g* for 20 min. The resultant pellet was washed three times, suspended in buffer at a protein concentration of 1–3 mg/ml, frozen and stored at −80 °C until use.

Binding to BB receptors

^{125}I-GRP (150–200 pM) and ^{125}I-[Tyr^4]BB (100–200 pM) were used interchangeably to label BB receptors on rat pancreatic membranes [16] using techniques described previously [12]. Binding to intact acini was determined by incubating acini in Krebs–Henseleit buffer containing 0.2% BSA and 1 mg of bacitracin/ml for 1 h at 37 °C. Bound and free ^{125}I-GRP were separated by vacuum filtration (Millipore Corp., Bedford, MA, U.S.A.) through Whatman GF/C filters, followed by washing with 3 × 5 ml of iso-osmotic cold Krebs–Henseleit buffer. Bound radioligand was measured by counting the radioactivity in an Isoflex γ-radiation counter at 75–80 % efficiency. Specific ^{125}I-GRP and ^{125}I-[Tyr^4]BB binding were defined with 1 μM-BB, and protein concentrations were chosen so less than 15 % of radioligand was bound at equilibrium. IC_{50} values (concns. causing 50 % inhibition of maximum binding) and Hill coefficients were calculated from Hill plots. Analysis of two binding sites was performed by non-linear iterative curve fitting [17]. Since the ^{125}I-GRP concentration used for competition curves (200 pM) was 10 % of its K_d value, the IC_{50} values of peptides approximated to their inhibition constants. Protein was determined using the Pierce protein assay (Pierce, Rockford, IL, U.S.A.).

Measurement of ^{3}H-labelled inositol phosphates

Minilobules were incubated for 1 h at 37 °C in 2 ml of Krebs–Henseleit medium in a silane-treated 25 ml Erlenmeyer flask containing 10–15 μCi of [^{3}H]inositol/ml. Labelled acini were washed three times with Krebs–Henseleit buffer, and 2 ml aliquots were placed into Erlenmeyer flasks. Minilobules were incubated for 30 min at 37 °C in Krebs–Henseleit medium containing 10 mM-LiCl and secretagogues. Incubations were terminated by addition of 1 ml of 2 mM-EDTA solution and the tissue was boiled for 5 min. Particulate material was sedimented by centrifugation and supernatants were collected for separation of inositol phosphates by h.p.l.c.

Samples (0.5 ml) of supernatant were injected into a Perkin–Elmer series 410 h.p.l.c., and inositol phosphates were separated on a 10 cm × 0.46 cm Zorbax anion-exchange column (DuPont, Boston, MA, U.S.A.) using the following ammonium formate (pH 3.75) elution gradient, pumped at 1.2 ml/min: 2 min, 100 % water; 20 min, linear gradient of 0–20 % ammonium formate; 25 min, linear gradient of 20–100 % 1 M-ammonium formate. Using this protocol, standard labelled inositol phosphates were eluted as follows: InsP_1, 12 min; InsP_2, 21 min; Ins(1,3,4)P_3, 31 min; Ins(1,4,5)P_3, 32 min; Ins(1,3,4,5)P_4, 37 min.

RESULTS

BB and neuromedins stimulate amylase secretion

NMC and NMB were full agonists of amylase secretin from pancreatic acini, producing similar maximum secretory responses to those evoked by BB (results not shown). Similar secretion maxima were also obtained with 1 μM-ranatensin, [Tyr^4]BB and GRP (results not shown). Bombesin and NMC exhibited EC_{50} values (concns. causing half-maximal effect) of 0.2 nM and 0.3 nM respectively, which were 10-fold lower than that of NMB (2 nM). This order of potency was similar to that determined by others in rat [10] and guinea pig [11] pancreas. Thus NMB was a significantly weaker agonist than BB, by 8–10-fold in the rat ([10]; the present study) and 170-fold in the guinea pig pancreas [11].

Stimulation of guinea pig pancreatic acini with BB resulted in desensitization of secretory responses to subsequent BB stimulation (Fig. 1). This desensitization was identical for BB and related peptides, and cholinergic responses were not significantly attenuated [18]. In order to test whether NMs and BB stimulate the same receptor in rat pancreas, we tested for cross-desensitization between BB and NMB. Fig. 1 and Table 1 show the results of exposing rat pancreatic acini to 10 nM of either BB or NMB followed by washing, a 30 min rest, and re-stimulating the acini with BB, NMB or acetylcholine. The control secretory

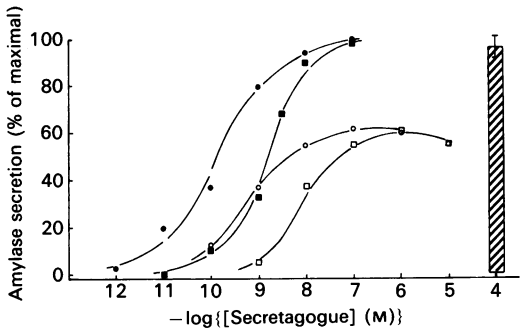

Fig. 1. Desensitization of BB- and NMB-stimulated amylase secretion by BB pre-treatment

Pancreatic acini were pre-incubated at 37 °C in the absence (●, ■) or the presence (○, □) of 10 nM-BB for 30 min, washed three times, allowed to rest for 30 min, re-washed twice and stimulated with the indicated concentrations of BB (●, ○), NMB (■, □) or acetylcholine (bar). Amylase release was measured over 30 min and secretion (calculated as % of BB control) was plotted against the concentration of secretagogue. The bar shows secretory responses (±S.E.M.) to acetylcholine (0.1 mM) following BB (10 nM) pre-treatment ($n = 3$). Results are representative of experiments performed three or four times.

Table 1. Effect of pre-treatment with BB and NMB on subsequent secretagogue-stimulated amylase release

Pancreatic acini were incubated with the indicated primary stimuli for 30 min at 37 °C and washed three times. Following a rest period of 30 min, acini were re-washed and stimulated with the secondary stimulus. Amylase release stimulated by the indicated concentrations of BB, NMB and acetylcholine was calculated as % of control values, which were 5.5±0.4%, 5.3±0.1% and 5.0±0.3% of total amylase/30 min respectively. Results show means±S.E.M. of three or four experiments.

	Amylase released by secondary stimulus (% of control)				
	BB		NMB		
Primary stimulus	0.01 μM	1 μM	0.01 μM	1 μM	Acetylcholine (100 μM)
None (control)	100	91±3	100	93±3	100
BB (10 nM)	52±4	61±1	35±4	60±2	96±5
NMB (10 nM)	74±2	76±2	45±3	71±3	91±3

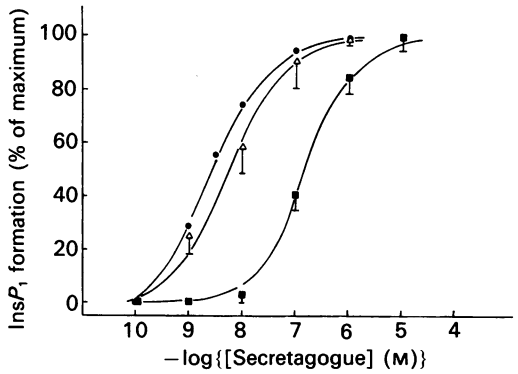

Fig. 2. Dose–response relationship of InsP_1 formation

InsP_1 generation is expressed as a percentage of maximal InsP_1 formation in the presence of BB. Results are means±S.E.M. of three different experiments. ●, BB; △, NMC; ■, NMB.

response and the responses following pre-incubation with BB are shown in Fig. 1. BB pre-treatment desensitized the subsequent response to BB to 60% of control values, and increased the EC_{50} value by 4-fold. Pre-treating acini with 10 nM-NMB resulted in less desensitization than that produced by BB (Table 1). This observation was consistent with lower occupancy of membrane ^{125}I-GRP-binding sites by 10 nM-NMB (77%) than by 10 nM-BB (91%). Secretion stimulated by subsequent maximal concentrations (1 μM) of BB and NMB were decreased similarly, to 76 and 71% of control values. These observations indicate that quantitatively similar cross-desensitization occurs between BB and NMB. Desensitization appeared to be confined to these peptides, since pretreatment with BB or NMB had little effect on subsequent stimulation by acetylcholine (Table 1).

Inositol phosphate formation

BB has been reported to stimulate inositol phosphate formation in a number of cells [19,20]. ^{3}H-labelled inositol phosphates formed in rat pancreatic acini following 30 min stimulation by bombesin (5 nM; in the presence of 10 mM-LiCl) were analysed by h.p.l.c. The results indicated the formation of InsP_1, InsP_2 and InsP_3, with the former being predominant (results not shown). Fig. 2 shows the dose–response curves for BB-, NMC- and NMB-stimulated InsP_1 formation. NMC and NMB produced maximum levels of InsP_1 which were not significantly different from those resulting from BB stimulation ($P > 0.05$). EC_{50} values for BB, NMC and NMB were 3, 5 and 141 nM respectively. Similar results were obtained for InsP_2 and InsP_3 formation (results not shown). The dose–response curves for BB- and NMC-stimulated InsP_1 formation were significantly (15–17-fold) right-shifted compared with their respective curves for amylase secretion (compare Fig. 2 with Fig. 1). These parameter differences were even greater for NMB (70-fold shift).

Inhibition by LL-BB

LL-BB is a selective BB receptor antagonist [13] which inhibits BB-stimulated amylase release from rat [12] and guinea pig [13] pancreatic acini. Table 2 shows the effects of LL-BB (10 μM) on BB-, NMC- and NMB-stimulated amylase release and inositol phosphate formation. LL-BB (10 μM) inhibited BB-, NMC- and NMB- (10 nM) stimulated amylase release by 67%, 78% and 98% respectively. These values were close to the expected values assuming competitive inhibition [12,13] and a dose ratio (ratio of equi-effective agonist concentrations for amylase secretion in the presence/absence of antagonist) for 10 μM-LL-BB of 200.

Table 2 also shows the effects of LL-BB (10 μM) on basal and secretagogue-stimulated inositol phosphate formation. LL-BB alone was unable to stimulate any significant InsP_1 production (34 c.p.m. above the control unstimulated InsP_1 levels of 24 c.p.m.), which correlates with its inability to stimulate amylase secretion [12,13]. However, LL-BB inhibited almost completely InsP_1 production stimulated by BB (10 nM), NMC (10 nM) or NMB (100 nM). Similar results were observed for InsP_2 and InsP_3 formation (results not shown). Inositol phosphate formation stimulated by 5 nM-BB was dose-dependently inhibited by LL-BB, with a calculated IC_{50} value of 125 nM (results not shown). This is similar to its IC_{50} values for inhibition of BB-stimulated amylase secretion (130 nM) and inhibition of ^{125}I-GRP binding to rat pancreatic membranes (106 nM) [12].

^{125}I-GRP binding

Membranes. ^{125}I-GRP bound to a single saturable population of sites on rat pancreatic membranes. Scatchard analysis showed that ^{125}I-GRP competition curves were linear, and calculated $B_{max.}$ and K_d values were 1168±140 fmol/mg of protein and 2.3±0.32 nM respectively ($n = 3$). The peptide agonists BB and NMC competed with ^{125}I-GRP binding to rat pancreatic membrane receptors at 22 °C with IC_{50} values of 0.5±0.10 and 0.4±0.1 nM respectively ($n = 7$) (see Table 4). NMB and GRP exhibited slightly lower IC_{50} values (1.8±0.4 and 2.6±0.6 nM respectively; $n = 6$–7). The Hill coefficients of the competition curves were close to one for BB (0.85±0.05) and GRP (1.06±0.06), and lower for NMC (0.73±0.06) and NMB (0.63±0.04) (see Table 4). These data suggest the existence of co-operative interactions or multiple affinity sites for NMC and NMB on pancreatic membranes.

Intact acini. Binding of BB congeners to intact pancreatic acini was determined in Krebs–Henseleit buffer for peptides in competition with ^{125}I-GRP. Table 3 compares the IC_{50} values of peptides measured at 37 °C and 22 °C, since binding studies of pancreatic membranes were conducted at the lower temperature. The K_d values for ^{125}I-GRP at 22 °C and 37 °C were 8.2±1.8 and 5.3±0.6 nM respectively; however, IC_{50} values of peptides for BB receptors on intact acini did not vary significantly at these temperatures. In all cases, the IC_{50} values of peptides for BB receptors on intact cells were higher than their respective values in membranes. This difference was least for GRP and BB (3–5-fold), but was more marked with NMC (19-fold) and NMB (68-fold). The IC_{50} values of peptides for occupation of intact pancreatic acinar cell receptors were similar to their EC_{50} values for

Table 2. Inhibition by LL-BB of peptide-stimulated amylase release and inositol phosphate formation

Rat pancreatic acini were stimulated with or without BB, NMB or NMC (10 nM) in the absence or the presence of LL-BB (10 μM) for 30 min, and the amount of amylase released into the medium was determined. [^{3}H]Inositol-labelled acini were stimulated with BB, NMB or NMC (10 nM) for 30 min in the absence or the presence of LL-BB (10 μM) in the presence of LiCl (10 mM), and InsP_1 formation was determined. Results are the means (±S.E.M.) of two or three experiments.

Treatment	Amylase release (% of total/30 min)	InsP_1 formed (c.p.m./30 min)
BB	5.6±0.4	3357
BB+LL-BB	1.4±0.1	71
NMC	5.9±0.2	2953
NMC+LL-BB	1.3±0.2	48
NMB	5.2±0.3	1048
NMB+LL-BB	1.1+0.2	51

Table 3. Effect of temperature on peptide binding to ^{125}I-GRP receptors on intact rat pancreatic acini

Competition of peptides for specific ^{125}I-GRP binding sites on intact pancreatic acini was measured at the indicated temperatures in Krebs–Henseleit buffer. IC_{50} values and Hill coefficients of the competition curves were calculated. Results are means ± S.E.M. of the indicated numbers of experiments.

	22 °C			37 °C		
Peptide	IC_{50} (nM)	h	n	IC_{50} (nM)	h	n
BB	2.6±0.3	1.04±0.04	3	1.9±0.3	0.93±0.06	4
GRP	8.4±1.3	0.94±0.06	3	7.4±1.1	1.06±0.06	4
NMC	7.7±0.9	0.93±0.05	3	7.6±1.2	0.88±0.06	3
NMB	122±23	0.94±0.06	3	324±55	0.88±0.05	6

stimulation of inositol phosphates. The competition curves of peptide binding to intact cells at 22 °C or 37 °C were steep, with Hill coefficients approaching unity, indicating a homogeneous population of binding sites for these agonists.

Guanine nucleotide regulation of binding

Receptor–G-protein coupling is often associated with guanine-nucleotide-modulated agonist binding [21]. The non-hydrolysable guanine nucleotide analogue Gpp[NH]p was examined for its effects on ^{125}I-GRP binding to rat pancreatic acinar membranes. However, we were unable to demonstrate a significant effect of Gpp[NH]p on the K_d of ^{125}I-GRP binding (control K_d, 2.3±0.03 nM; Gpp[NH]p-treated, 3.5±0.07 nM), or on its $B_{max.}$ (control, 1168±140 fmol/mg; Gpp[NH]p-treated, 927±153 fmol/mg of protein). Simulation of binding data indicated the difficulty of discriminating small shifts in GRP competition curves which are indicative of Gpp[NH]p-mediated effects on the K_d. In order to examine more directly Gpp[NH]p modulation of agonist binding to BB receptors, we measured the effects of Gpp[NH]p on ^{125}I-[Tyr^4]BB dissociation from rat pancreatic membranes. Fig. 3 shows the kinetics of dissociation on addition of excess BB (1 μM) to membranes labelled at steady state with ^{125}I-[Tyr^4]BB. ^{125}I-[Tyr^4]BB dissociated slowly, and only 25% of the specifically bound radioligand was lost after 60 min. ^{125}I-[Tyr^4]BB binding was not irreversible, since subsequent addition of Gpp[NH]p after 45 min produced further dissociation (Fig. 3). In the co-presence of Gpp[NH]p and BB

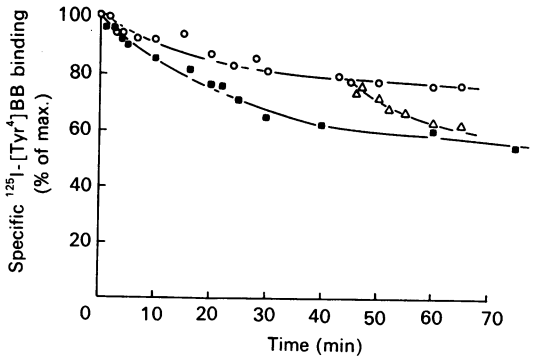

Fig. 3. ^{125}I-[Tyr^4]BB dissociation kinetics from pancreatic membranes in the absence and the presence of Gpp[NH]p

Acinar membranes were incubated with ^{125}I-[Tyr^4]BB (133 pM) for 60 min at 22 °C. BB (1 μM) was added to initiate dissociation (○) and Gpp[NH]p (5 μl, 2 mM) was added concurrently with BB (■) or after 45 min (△). Membranes were filtered at the indicated times and receptor-bound ^{125}I-[Tyr^4]BB was measured. A parallel series of tubes was incubated with 1 μM-BB to define non-specific binding, and specific binding was calculated and expressed as a percentage of maximal binding. Results show a representative experiment conducted three times with similar results.

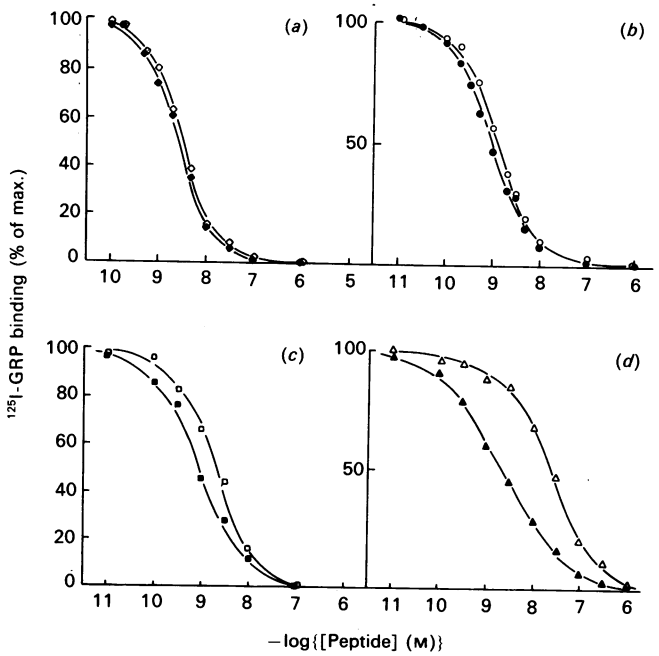

Fig. 4. Peptide binding to rat pancreatic membranes in the absence or the presence of Gpp[NH]p

Acinar membranes were incubated at 22 °C with ^{125}I-GRP and the indicated concentrations of peptides in the absence (closed symbols) or presence (open symbols) of 100 μM-Gpp[NH]p. Specific binding was determined and plotted as a percentage of the maximum. Results show mean curves of experiments performed 3–7 times. (*a*) GRP, (*b*) BB, (*c*) NMC, (*d*) NMB.

the rate of dissociation was significantly faster than with BB alone, although only 40% of bound ligand could be dissociated in 60 min. similar results were obtained with ^{125}I-GRP as the radioligand (results not shown).

Fig. 4 shows the effects of Gpp[NH]p on the competition curves of GRP, BB, NMC and NMB measured at 22 °C. BB competition curves, like those of GRP, were little affected by Gpp[NH]p, whereas NMC and NMB curves were right-shifted

Table 4. Effects of Gpp[NH]p and temperature on peptide binding to ^{125}I-GRP binding sites on rat pancreatic membranes

Competition experiments were conducted at 22 °C or 37 °C in the absence or the presence of 100 μM-Gpp[NH]p, and IC_{50} values and Hill coefficients were determined from iterative curve fitting. Results show means ± S.E.M. of experiments performed 3–7 times.

	22 °C				37 °C			
	Control		+Gpp[NH]p		Control		+Gpp[NH]p	
Peptide	IC_{50} (nM)	h	IC_{50} (nM)	h	IC_{50} (nM)	h	IC_{50} (nM)	h
BB	0.5±0.1	0.85±0.05	0.7±0.1	0.86±0.06	0.6±0.1	0.72±0.06	1.8±0.3	0.93±0.05
GRP	2.6±0.6	1.06±0.06	2.8±0.5	1.10±0.10	2.6±0.4	1.04±0.05	4.9±0.8	1.15±0.1
NMC	0.4±0.1	0.73±0.06	1.4±0.2	0.77±0.06	1.2±0.5	0.61±0.07	10±2	0.95±0.05
NMB	1.8±0.4	0.63±0.04	29±3	0.75±0.05	48±5	0.71±0.05	152±20	0.95±0.06

Table 5. Effects of NaCl and Gpp[NH]p on peptide binding to rat pancreatic membranes

Competition experiments were conducted with ^{125}I-[Tyr^4]BB (100–200 pM) at 22 °C in the absence or the presence of NaCl (40 mM) or both NaCl and Gpp[NH]p (0.1 mM). Results show mean ± S.E.M. IC_{50} values and Hill coefficients from 3–7 experiments.

	IC_{50} (nM)			h		
Peptide	Control	+NaCl	+NaCl+Gpp[NH]p	Control	+NaCl	+NaCl+Gpp[NH]p
BB	0.7±0.1	0.9±0.1	4.0±1.0	0.92±0.10	0.81±0.11	0.91±0.08
GRP	1.7±0.6	2.3±0.3	6.8±1.5	1.16±0.12	1.04±0.05	1.02±0.04
NMB	2.2±0.4	13±4	700±30	0.58±0.06	0.82±0.08	0.90±0.10

by 3.5- and 16-fold respectively. Hill coefficients of NMB competition curves in the presence of Gpp[NH]p were steeper than in its absence (control, 0.63; Gpp[NH]p-treated, 0.75) (Table 4). Two-site analysis of the NMB competition curve gave a significantly better fit than the one-site analysis [control, K_1 = 0.5 nM (60%), K_2 = 17 nM (40%); +Gpp[NH]p, K_1 = 0.4 nM (19%), K_2 = 48 nM (81%), $P < 0.05$]. Similar two-site analysis of the NMC competition curve gave the following parameters: control, K_1 = 0.2 nM (67%), K_2 = 2.9 nM (33%); +Gpp[NH]p, K_1 = 0.2 nM (35%), K_2 = 3.3 nM (65%). Analysis of GRP and BB competition curves by a two-site model did not indicate a better fit than a one-site model ($P > 0.4$). These results clearly show differences in the ability of Gpp[NH]p to regulate binding of BB and the neuromedins to rat pancreatic acinar membranes. Guanine nucleotides appear to significantly decrease the proportion of high-affinity NMB- and NMC-binding sites.

We have examined the effect of Gpp[NH]p on the competition curves of other structurally related peptides. The binding of the decapeptide NMB and its 30-amino-acid-containing precursor peptide were most sensitive to the guanine nucleotide. The ratio of the IC_{50} values of peptides measured at 22 °C in the presence and absence of Gpp[NH]p were (in order of guanine nucleotide sensitivity): NMB (16) > NMB-30 (8) > litorin (3.5) = NMC (3.5) > BB (1.4) > ranatensin (1.5) > [Tyr^4]BB = GRP (10).

Effect of temperature and NaCl on peptide binding to membranes

Since our measurements of secretion and phosphoinositide breakdown were conducted in physiological media at 37 °C, we examined the influence of temperature and NaCl on peptide binding to pancreatic membranes. Table 4 compares the IC_{50} values and Hill coefficients of peptides in competition with ^{125}I-GRP binding to pancreatic membranes obtained at 22 °C and 37 °C. Elevated temperature markedly increased the IC_{50} value of NMB (27-fold), but gave smaller increases for NMC (3-fold) and negligible changes for BB and GRP. These effects were quantitatively similar to those produced by Gpp[NH]p at 22 °C, although Hill coefficients were less than one for all peptides except GRP. However, analysis of binding curves using a two-site model did not indicate a significantly better fit than a one-site model ($P > 0.1$). At 37 °C, Gpp[NH]p modulated all competition curves and in its presence Hill coefficients were not significantly different from one, indicating a homogeneous population of sites.

NaCl produced dose-dependent inhibition of ^{125}I-[Tyr^4]BB binding to pancreatic membranes. The IC_{50} for this inhibition was 40 mM, and 150 mM-NaCl inhibited binding by 73%. The effects of 40 mM-NaCl on the IC_{50} values of peptides in competition with ^{125}I-[Tyr^4]BB binding are shown in Table 5. NaCl produced quantitatively similar effects to those of Gpp[NH]p, resulting in small decreases in the affinities of BB and GRP but in a pronounced decrease in NMB affinity (compare Table 5 with Table 4). Kinetic studies also showed quantitatively similar dissociation of ^{125}I-[Tyr^4]BB from labelled membranes exposed to either NaCl (40 mM) or Gpp[NH]p (100 μM) (results not shown).

The combination of NaCl and Gpp[NH]p produced marked decreases in ^{125}I-[Tyr^4]BB binding (74±10%; n = 8) and pronounced increases in IC_{50} value for all peptides. The decrease in affinity was again more significant for NMB (54-fold) than for GRP or BB (3–8-fold). Dissociation of ^{125}I-[Tyr^4]BB from pancreatic membranes by 1 μM-BB in the presence of NaCl and Gpp[NH]p was rapid and almost complete after 60 min (results not shown). The IC_{50} values obtained for BB, GRP and NMB binding to membranes in the presence of NaCl and Gpp[NH]p approximated to values obtained with intact pancreatic acini (compare Table 5 with Table 3).

Effect of pertussis toxin on peptide binding

Pertussis-toxin-insensitive G-proteins have been implicated in the binding of BB to a number of cell types [16,22]. Since binding

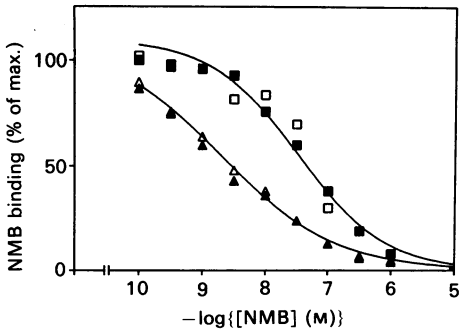

Fig. 5. Effect of pertussis toxin treatment on Gpp[NH]p modulation of NMB binding to pancreatic membranes

Intact acini were incubated in the absence or the presence of 1 μg of pertussis toxin/ml for 4 h at 37 °C, then membranes were prepared and ^{125}I-GRP binding was determined using the indicated concentrations of NMB in the absence (△, control; ▲, pertussis toxin) or presence of 100 μM-Gpp[NH]p (□, control; ■, pertussis toxin). Results show representative curves of experiments performed three times.

of NMB but not BB to pancreatic membranes was sensitive to guanine nucleotide regulation, we investigated the toxin sensitivity of this guanine nucleotide regulation. Pancreatic acini were treated with activated pertussis toxin (1 μg/ml) for 4 h at 37 °C, then membranes were prepared and competition curves were determined in the absence and the presence of Gpp[NH]p. Fig. 5 shows that Gpp[NH]p modulated the binding of NMB to control membranes from vehicle-treated cells in an analogous fashion to that demonstrated previously (see Fig. 4). Pertussis toxin treatment produced no significant effect on ^{125}I-[Tyr^4]BB binding (results not shown), nor did it influence the ability of the guanine nucleotide to modulate the affinity of NMB for the membranes. Moreover, toxin treatment had no effect on the competition curves of BB (results not shown). These data suggest that guanine nucleotide regulation of BB receptors occurs via a pertussis-toxin-insensitive G-protein.

DISCUSSION

We have previously proposed the existence of different subtypes of BB receptors based on studies with frog oesophageal peptic cells and rat pancreatic acini [12]. More recently, Von Schrenck *et al.* [11] reported that the affinity of NMB for rat oesophageal BB receptors was 250-fold higher than that for BB receptors in rat pancreas. The major objective of the present study was to determine whether BB and NMB interact with the same or different receptor subtypes in the pancreas.

Consistent with earlier studies [1], we have demonstrated the presence of a BB receptor in rat pancreas, and stimulation of this receptor was accompanied by amylase secretion (Fig. 1). Analogous to the situation with BB receptors in other tissues such as Swiss 3T3 cells [19,23] and HIT-T15 [24] cells, stimulation of the BB receptor in the pancreas was accompanied by phosphoinositide breakdown. A possible sequence of events leading to amylase release would involve receptor occupancy, phosphoinositide breakdown, inositol trisphosphate and diacylglycerol generation, Ca^{2+} release and mobilization. Our findings that BB, NMC and NMB each stimulated inositol phosphate accumulation to the same maximal extent, and that pre-treatment with either BB or NMB desensitized the response to both of these agonists to the same degree, while having no effect on the muscarinic receptor response, indicated that both BB and NMB most likely interacted with the same receptor.

Although BB and NMB appeared to bind to the same receptor in the pancreas, it was notable that they demonstrated differential sensitivities to guanine nucleotides. When binding studies were carried out with pancreatic membranes at 22 °C, Gpp[NH]p had little effect on GRP or BB competition curves, whereas it shifted the NMB displacement curve by a factor of 10. Modulation of BB binding by guanine nucleotides has been suggested as evidence for the involvement of receptor–G-protein coupling [16]. The degree of guanine nucleotide modulation of agonist binding has been correlated with the relative efficacy of muscarinic agonists to stimulate phosphoinositide breakdown and Ca^{2+} mobilization [25]. However, although the binding of BB and NMB to pancreatic membranes differed markedly in their sensitivities to guanine nucleotides, we cannot account for this by a diminished efficacy of BB in stimulating amylase secretion or inositol phosphate formation. A lack of guanine nucleotide regulation of BB binding to brain membranes has also been reported [26], although these results may have been due to the use of low incubation temperatures [16]. Since our binding studies were conducted at elevated temperatures, the modest effects of guanine nucleotides on BB and GRP binding cannot be attributed to temperature limitations on a receptor–G-protein interaction. A lack of guanine nucleotide regulation of angiotensin binding to the AT_2 receptor has also been suggested as evidence the absence of receptor–G-protein coupling [27]. However, evidence from a number of laboratories has implicated a pertussis-toxin-insensitive G-protein in the coupling of the BB receptor to phospholipase C in certain tissues [16,19,22,28]. The present study in pancreas has confirmed the lack of pertussis toxin effects on guanine nucleotide regulation of NMB binding. These data suggest that the G-protein involved in coupling the pancreatic BB receptor to amylase secretion is also pertussis-toxin-insensitive.

Our finding of differential guanine nucleotide regulation of NMB and BB binding may also be interpreted as evidence that BB and NMB, while binding to the same receptor, result in receptor coupling to different G-proteins. An example of a single receptor coupling to two different effector systems in the pancreas has recently been provided by Saluja *et al.* [29]. They found that although both cholecystokinin (CCK) and CCK-JMV-180 [a synthetic analogue of CCK with the structure Boc-Tyr(SO_3)-Nle-Gly-Trp-Nle-Asp-2-phenyl ester] stimulated amylase secretion by interacting with CCK receptors, only CCK stimulated inositol phosphate formation. It is clear that the BB receptor is coupled to a number of effector systems, which may involve different G-proteins. Thus, in 3T3 cells, pertussis toxin inhibited BB-stimulated DNA synthesis, but had no effect on phosphoinositide turnover [22]. BB has also been shown to cause rapid release of arachidonic acid and prostaglandins from Swiss 3T3 cells [30].

We also observed in the present study that peptides exhibited several-fold lower affinities for BB receptors on intact acini than on membranes at 22 °C. Elevation of the temperature to 37 °C and inclusion of guanine nucleotide increased the IC_{50} values of peptides for BB receptors on membrane preparations. The presence of NaCl also produced quantitatively similar decreases in the affinities of peptides to those produced by elevated temperature or guanine nucleotides. These effects are probably related to the ability of these interventions to enhance the dissociation rate of ^{125}I-[Tyr^4]BB from the receptor. Thus the co-presence of NaCl and guanine nucleotides resulted in peptide binding constants for membrane receptors which were similar to the values determined in intact cells. The effects of NaCl on BB binding to pancreatic receptors were similar to the findings of BB receptor binding in rat brain membranes [31].

The competition curves for NMC and NMB were best described by a two-site model. Binding to the high-affinity site

was decreased but not eliminated by guanine nucleotides at 22 °C, which suggested that Gpp[NH]p mediated the breakdown of ternary receptor–peptide–G-protein complex [32]. Fischer & Schonbrunn [16] have also reported the partial conversion of high-affinity ^{125}I-[Tyr^4]BB sites to a rapidly dissociating state in GH_4C_1 membranes. Since all peptide competition curves at 37 °C in the presence of Gpp[NH]p were monophasic, it is likely that at this temperature Gpp[NH]p induced the complete conversion of high-affinity agonist-induced BB receptor states to a uniform low-affinity state. The physiological significance of these high-affinity peptide-agonist-induced states generated at 22 °C in non-physiological buffers remains to be elucidated. It is possible that the difference in the curves for functional (secretory) responses and for receptor occupancy measured in membranes is related to spare receptor capacity for the agonist [33]. According to this concept, NMB would have the largest spare receptor capacity, producing substantial amylase secretion with little receptor occupancy, and with very little inositol phosphate formation. Ek & Nahorski [34] have shown markedly different relationships between inositol phosphate formation and occupancy of muscarinic receptors on membrane preparations from different tissues, which was attributed to more efficient coupling of parotid receptors to phosphoinositide turnover. However, it is difficult to attribute our findings to differences in the stoichiometry of receptors, G-protein or phospholipase C, as we were dealing with only one tissue.

It does remain a possibility, however, that BB-related peptides exhibit different efficiencies in receptor–effector coupling. By contrast with data obtained with membrane preparations, occupancy of BB receptors on intact cells correlated well with the dose–response curves for inositol phosphate formation, suggesting little spare receptor capacity for all peptides tested in producing phosphoinositide breakdown. However, the binding constants obtained for peptide interactions with intact cells at equilibrium clearly reflect the results of numerous processes, including binding, receptor internalization, etc. Thus the high-affinity peptide sites seen in membranes may reflect rapidly induced states (sites) in whole cells, which are not demonstrable at equilibrium. Non-equilibrium binding of catecholamines to intact cells indicates the existence of such high-affinity agonist-induced β-adrenergic receptor sites [35,36]. The physiological significance of the high-affinity sites and their relationship to inositol phosphate formation and amylase secretion remain to be established.

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