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# Characterization of Bombesin Receptors Using a Novel, Potent, Radiolabeled Antagonist That Distinguishes Bombesin Receptor Subtypes

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**SUMMARY**

Bombesin (Bn)-related peptides affect numerous cell functions; however, receptor characterization by radiolabeled ligands is limited because only radiolabeled agonists exist. In the present study we demonstrate that [D-Tyr$^6$]Bn(6–13)methyl ester [[D-Tyr$^6$]Bn(6–13)ME] functions as a Bn receptor antagonist with high affinity. The binding of both the radiolabeled agonist $^{125}$I-[Tyr$^4$]Bn and the radiolabeled antagonist $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME to AR42J cells, murine 3T3 cells, and dispersed guinea pig pancreatic acini was time and temperature dependent, saturable, and reversible. Binding of the antagonist more rapidly reached equilibrium and was more rapidly reversible. Guanine nucleotides did not affect binding of the radiolabeled antagonist, whereas guanosine-5′-($\beta,\gamma$-imido)triphosphate decreased agonist binding by decreasing Bn receptor affinity. Acid stripping studies demonstrated that the radiolabeled agonist, but not the antagonist, was internalized in each cell system. Bn receptor affinities for various Bn receptor agonists or antagonists in each cell system were identical when computed from an analysis of inhibition curves for binding of radiolabeled agonist or antagonist. However, with AR42J cells and 3T3 cells the radiolabeled agonist demonstrated a >2-fold higher number of Bn receptors than did the radiolabeled antagonist. Binding studies using cell membranes, in contrast to cells, showed equal numbers of Bn receptors with either radiolabeled ligand. The radiolabeled agonist demonstrated high affinity binding to both rat pancreatic acinar and esophageal muscularis mucosa membranes, whereas the radiolabeled antagonist interacted with high affinity only with the gastrin-releasing peptide-preferring subtype of Bn receptors on pancreatic tissue. These results demonstrate that $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME is a high affinity radiolabeled antagonist that interacts specifically with Bn receptors. In contrast to the radiolabeled agonist, binding of the antagonist is not affected by guanine nucleotides and it is not internalized, which allows quantitation of only Bn cell surface receptors. Furthermore, the radiolabeled antagonist can distinguish Bn receptor subtypes, whereas the radiolabeled agonist does not. This ligand should prove useful for characterizing Bn receptors as well as studying their regulation.

Bn and the related mammalian peptides GRP, GRP-18–27 (NMC), and NMB have been shown to produce a wide range of biological responses in mammalian tissues (1). These include potent effects in the CNS such as thermoregulation (2), chemotaxis (3), stimulation of the release of numerous gastrointestinal peptides (4), smooth muscle contraction (5), increased satiety (6), and stimulation of pancreatic enzyme secretion (7). Bn-related peptides also function as growth factors in various normal and tumor cells (8–10) and as autocrine growth factors in small cell lung cancer cells (9, 11).

Recent studies demonstrate the widespread occurrence of specific Bn receptors on a number of tumor cell lines (8, 12–16), in gastrointestinal tissues (1, 17), and throughout the CNS (18, 19). Binding and functional studies suggested that there may exist more than one Bn receptor subtype (5, 20–23), and recent cloning studies demonstrated two Bn receptor subtypes, a receptor with high affinity for GRP (the GRP receptor) and one with high affinity for NMB (the NMB receptor) (24–26). To date, all binding studies characterizing these receptors, similarly to the case for receptors for most gastrointestinal peptides, have been performed using radiolabeled agonists, either radiolabeled [D-Tyr$^4$]Bn, GRP, or NMB (11–17). A number of these studies suggest that these radiolabeled Bn agonists are internalized in some cells (14, 15, 27, 28), their binding is

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**ABBREVIATIONS:** Bn, bombesin; GRP, gastrin-releasing peptide; NMB, neuromedin B; NMC, neuromedin C; CNS, central nervous system; BSA, bovine serum albumin; Gpp(NH)p, guanosine-5′-($\beta,\gamma$-imido)triphosphate; EGTA, ethylene glycol bis-($\beta$-aminoethyl ether)-*N,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GnRH, gonadotropin-releasing hormone; TEAP, tetraethylammonium phosphate; ME, methyl ester; HPLC, high performance liquid chromatography; CCK, cholecystokinin; LHRH, luteinizing hormone-releasing hormone.

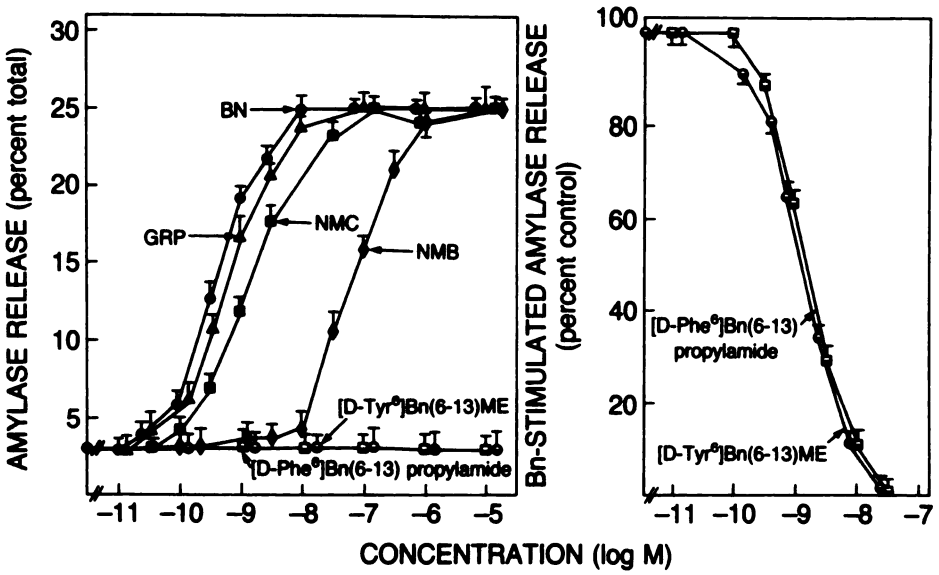


**Fig. 1.** Ability of various Bn-related peptides to stimulate (*left*) or inhibit (*right*) Bn-stimulated amylase release from dispersed acini from guinea pig pancreas. Pancreatic acini were incubated with the indicated concentration of each Bn-related peptide alone (*left*) or with 0.3 nM Bn and the indicated concentration of [D-Phe⁶]Bn(6–13)propylamide or [D-Tyr⁶]Bn(6–13)ME (*right*). *Left*, amylase release is expressed as the percentage of the total cellular amylase released into the extracellular medium during the incubation. *Right*, amylase release is expressed as the percentage of the stimulated amylase release caused by 0.3 nM Bn alone; basal amylase release was 3.5 ± 1.2% and 0.3 nM Bn-stimulated amylase release was 17.3 ± 2.3% of the total cellular amylase. Each *point* is the mean of four experiments and in each experiment each value was determined in duplicate. *Vertical bar*, 1 SE.

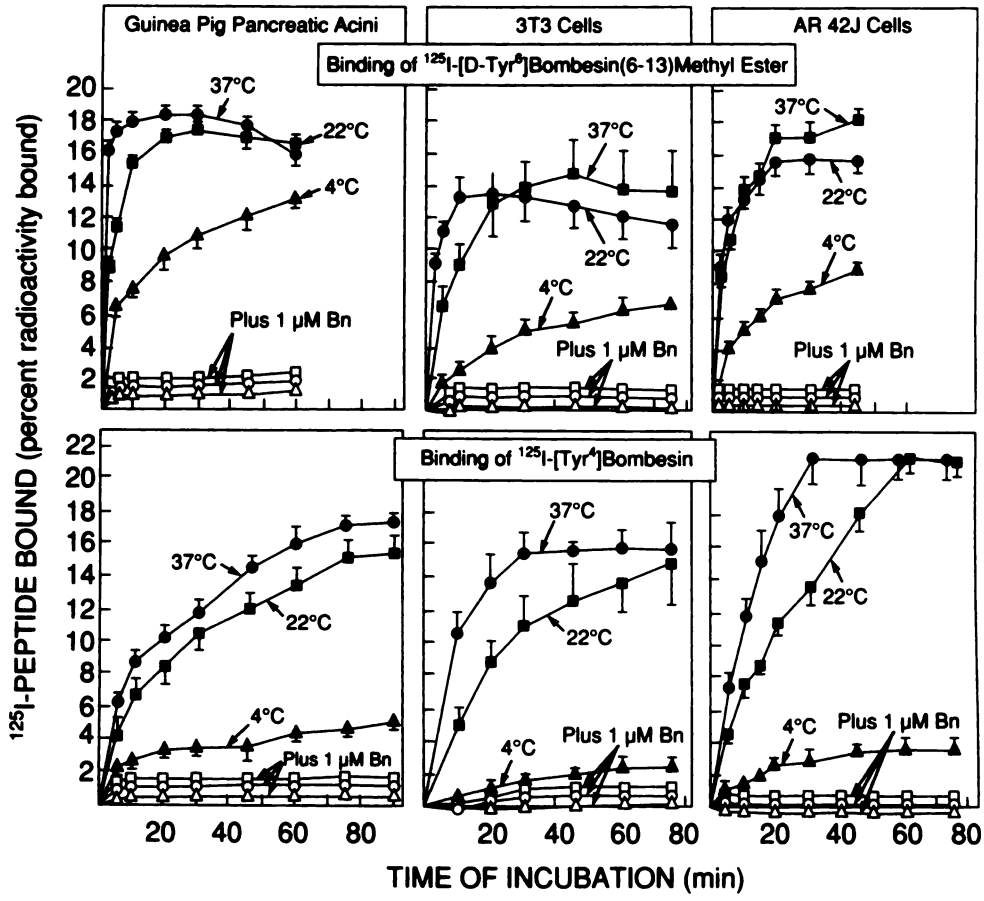


**Fig. 2.** Time and temperature dependence of binding of the antagonist $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME (*top*) and the agonist $^{125}$I-[Tyr$^4$]Bn (*bottom*) to guinea pig pancreatic acini (*left*), 3T3 cells (*middle*), or AR42J cells (*right*). Guinea pig pancreatic acini, 3T3 cells, or AR42J cells were incubated with 50 pM $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME or 50 pM $^{125}$I-[Tyr$^4$]Bn alone (*closed symbols*) or with 1 μM Bn (*open symbols*) at the indicated temperatures and for the indicated times. Results are expressed as the percentage of the added counts bound. In each experiment each value was determined in duplicate and results given are means of at least four separate experiments. *Vertical bar*, 1 SE.

affected by guanine nucleotides (28–30), and the agonist can cause desensitization, which could affect receptor numbers or affinity (31–33). Furthermore, results with some radiolabeled agonists such as radiolabeled [Tyr$^4$]Bn may be limited because they may not distinguish receptor subtypes (20). Studies of receptors for muscarinic cholinergic agents, α- and β-adrenergic agents, and GnRH have shown that there are advantages to characterizing receptors using radiolabeled antagonists as well as radiolabeled agonists (34–43). Radiolabeled antagonists often have the advantage of greater selectivity in distinguishing various receptor subtypes, they do not cause desensitization and thus their presence does not change receptor number or affinity, their binding may not be affected by guanine nucleotides and therefore they may be more useful for assays of solubilized receptors, and they may not be internalized, allowing quantitation of cell surface receptors.

Recently a number of classes of Bn receptor antagonists have been described, some with affinities in the nanomolar range

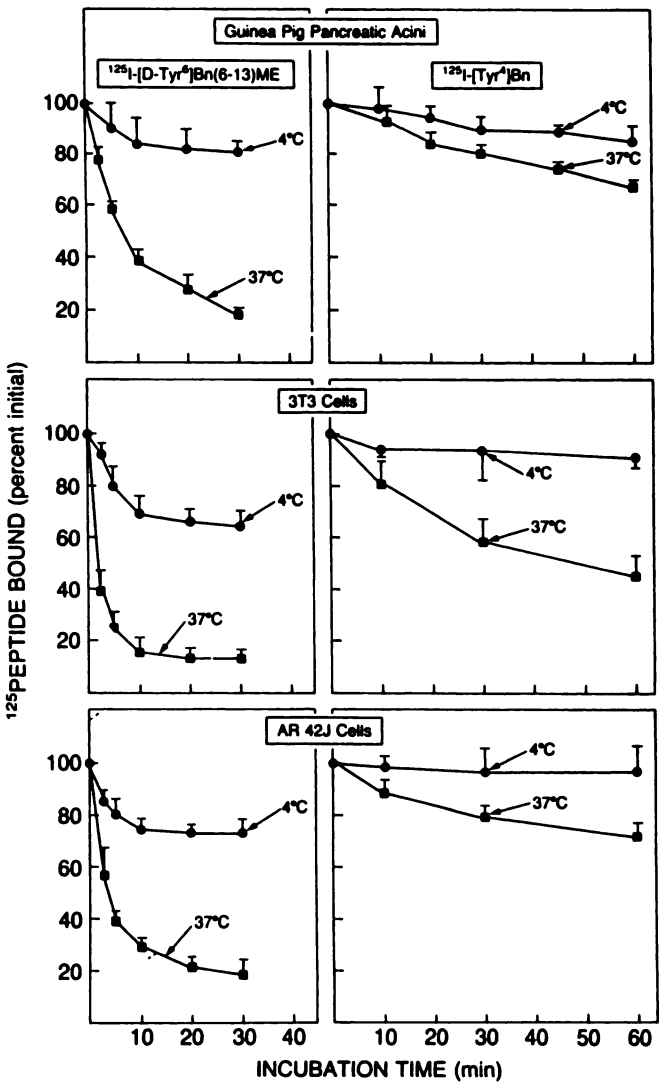


**Fig. 3.** Dissociation of the bound antagonist $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME (*left*) and the bound agonist $^{125}$I-[Tyr$^4$]Bn (*right*) from guinea pig pancreatic acini (*top*), 3T3 cells (*middle*), or AR42J cells (*bottom*). Guinea pig pancreatic acini, murine Swiss 3T3 cells, or AR42J cells were incubated at 37° with 50 pM $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME for 20 min or with 50 pM $^{125}$I-[Tyr$^4$]Bn for 60 min. At that time 100-ml aliquots were added to 5 ml of standard incubation buffer at 4° or 37° and incubated at the indicated temperatures for the indicated times. The samples were filtered over GF/B filters. The results are expressed as the percentage of saturable binding at the beginning of the second incubation. In each experiment each value was determined in duplicate and each *point* is the mean of at least three experiments. *Vertical bar*, 1 SE.

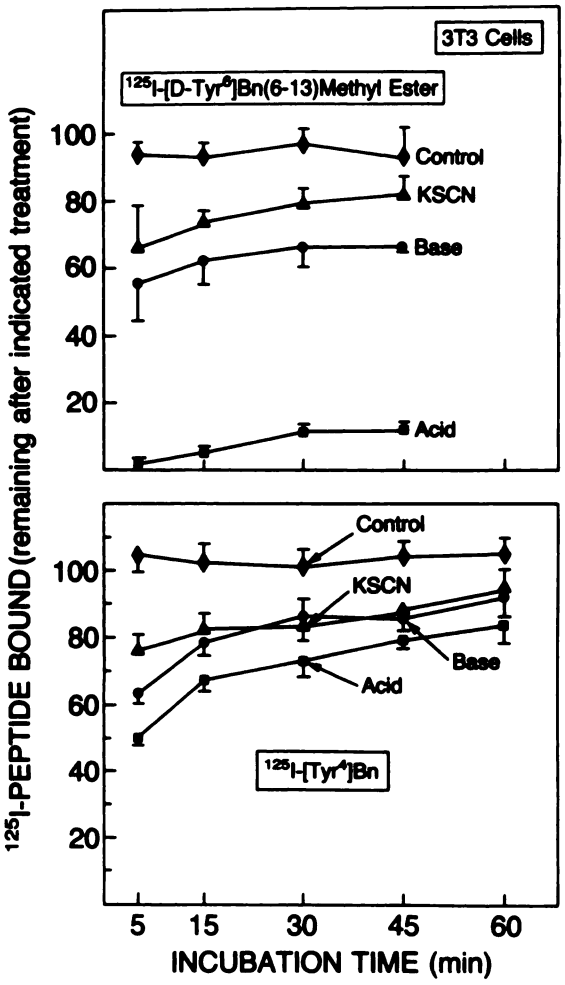


**Fig. 4.** Effect of incubation time with each ligand on the ability of acid, base, or KSCN treatment (stripping) to remove the surface-bound antagonist $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME or agonist $^{125}$I-[Tyr$^4$]Bn from 3T3 cells. Murine Swiss 3T3 cells were incubated at 37° with 50 pM $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME (*top*) or 50 pM $^{125}$I-[Tyr$^4$]Bn (*bottom*) for the various incubation times indicated. Aliquots (100 μl) were then added to 0.2 M acetic acid with 0.5 M NaCl (pH 2.5) (*Acid*), 0.2 M glycine-NaOH with 0.5 M NaCl (pH 10.5) (*Base*), 0.5 M potassium thiocyanate (*KSCN*), or standard incubation buffer (*Control*), for 5 min at 4°. The results are expressed as the percentage of control saturable binding remaining after the indicated stripping treatment performed at the various times of incubation with each ligand. Data represent means of at least three separate experiments. *Vertical bar*, 1 SE.

(23, 44–47), and some are reported to have different affinities for Bn receptors on different tissues, suggesting that they may distinguish receptor subtypes (22, 23). In the present study we have developed a radiolabeled antagonist that has high affinity for Bn receptors, and we compare its ability to interact with Bn receptors in a number of different tissues with that of a radiolabeled agonist.

## Experimental Procedures

### Materials

NIH-Hartley strain guinea pigs (150–200 g) were obtained from the Small Animals Section, Veterinary Resources Branch, National Institutes of Health. Leupeptin, BSA (fraction V), and HEPES were from Boehringer-Mannheim Biochemicals (Indianapolis, IN); purified collagenase (type CLSPA, 440 units/mg) from Worthington Biochemicals (Freehold, NJ); soybean trypsin inhibitor, EGTA, chymostatin, and bacitracin from Sigma Chemical Co. (St. Louis, MO); essential vitamin mixture (100× concentrated) from Microbiological Associates (Bethesda, MD); glutamine from Research Plus Laboratories (Bayonne, NJ); Na$^{125}$I (14.1 mCi/μg) from Amersham Searle (Arlington Heights, IL); 1,3,4,6-tetrachloro-3a,6a-diphenylglycouril (Iodo-Gen) from Pierce Chemical Co. (Rockford, IL); Phadebas amylase test reagent from Pharmacia Diagnostics (Piscataway, NJ); GRP-18–27 (NMC), NMB, Bn, and [Tyr$^4$]Bn from Peninsula Laboratories (Belmont, CA); Gpp(NH)p tetralithium salt from Fluka Chemical Corp. (Ronkonkoma, NY); Tris from Bethesda Research Laboratories (Gaithersburg, MD); and benzamidine hydrochloride from Eastman Kodak Co. (Rochester, NY). Protected amino acids and other synthetic reagents were obtained from Advanced Chem Tech (Louisville, KY).

TABLE 1

**Acid strippability of the bound antagonist $^{125}$I-[D-Tyr$^6$]Bn(6-13)ME or the bound agonist $^{125}$I-[Tyr$^4$]Bn from guinea pig pancreatic acini, 3T3 cells, or AR42J cells**

Guinea pig pancreatic acini, 3T3 cells, or AR42J cells were incubated with the agonist (50 pM $^{125}$I-[Tyr$^4$]Bn) or the antagonist [50 pM $^{125}$I-[D-Tyr$^6$]Bn(6-13)ME] at 37° for the indicated time, and then acid strippability of bound ligand was determining using 0.2 M acetic acid and 0.5 M NaCl (pH 2.5), as described in Experimental Procedures. Binding time refers to the time the indicated cell type was incubated with 50 pM $^{125}$I-[Tyr$^4$]Bn or $^{125}$I-[D-Tyr$^6$]Bn(6-13)ME before acid stripping. Results are expressed as the percentage of control saturable binding (incubation with buffer) remaining after ligand stripping during a 5-min incubation at 4° with 0.2 M acetic acid and 0.5 M NaCl (pH 2.5). Data represent means ± 1 SE from at least four separate experiments.

| Cell type | Binding time | $^{125}$I-Peptide bound | |
|---|---|---|---|
| | | $^{125}$I-[D-Tyr$^6$]Bn(6-13)ME | $^{125}$I-[Tyr$^4$]Bn |
| | *min* | *% acid resistant* | |
| 3T3 cells | 10 | 2.0 ± 0.4 | 64 ± 4 |
| | 60 | 13.0 ± 3.5 | 83 ± 5 |
| AR42J cells | 10 | 4.0 ± 0.2 | 30 ± 3 |
| | 60 | 8.0 ± 1.0 | 36 ± 2 |
| Guinea pig pancreatic acini | 10 | 1.2 ± 0.1 | 22 ± 3 |
| | 60 | 9.3 ± 0.4 | 23 ± 1 |

TABLE 2

**Effect of various agents on binding of the antagonist $^{125}$I-[D-Tyr$^6$]Bn(6-13)ME to guinea pig pancreatic acini, AR42J cells, or 3T3 cells**

Each of the three different cell systems was incubated with 50 pM $^{125}$I-[D-Tyr$^6$]Bn(6-13)ME, either alone, with 1 μM Bn, or with the indicated concentration of the agent listed. Results are expressed as the percentage of the saturable binding with no unlabeled agent added. Results are means ± 1 SE from three separate experiments.

| Agent added[a] | Binding of $^{125}$I-[D-Tyr$^6$]Bn(6-13)ME | | |
|---|---|---|---|
| | Guinea pig pancreatic acini | AR42J cells | 3T3 cells |
| | *% of control* | | |
| Bn (0.1 μM) | 4 ± 1[b] | 14 ± 1[b] | 1 ± 1[b] |
| GRP (0.1 μM) | 2 ± 1[b] | 16 ± 2[b] | 6 ± 3[b] |
| GRP-18-27 (0.1 μM) | 4 ± 1[b] | 24 ± 1[b] | 32 ± 11[b] |
| NB (0.1 μM) | 27 ± 7[b] | 47 ± 1[b] | 22 ± 5[b] |
| [D-Phe$^6$]Bn(6-13)PA (0.1 μM) | 3 ± 1[b] | 3 ± 2[b] | 2 ± 1[b] |
| [D-Tyr$^6$]Bn(6-13)ME (0.1 μM) | 2 ± 1[b] | 2 ± 1[b] | 2 ± 1[b] |
| Substance P (1 μM) | 88 ± 3 | 101 ± 1 | 97 ± 4 |
| Gastrin-17-I (1 μM) | 102 ± 2 | 95 ± 2 | 93 ± 3 |
| CCK-8 (1 μM) | 93 ± 2 | 96 ± 5 | 92 ± 2 |
| Carbachol (0.1 μM) | 91 ± 4 | 95 ± 1 | 90 ± 2 |
| VIP (1 μM) | 95 ± 6 | 99 ± 3 | 98 ± 11 |
| Secretin (1 μM) | 93 ± 2 | 97 ± 2 | 93 ± 6 |
| CGRP (1 μM) | 108 ± 7 | 97 ± 3 | 99 ± 11 |

[a] CCK-8, carboxyl-terminal octapeptide of CCK; VIP, vasoactive intestinal peptide; CGRP, calcitonin gene-related peptide; PA, propylamide.
[b] $p < 0.01$, compared with saturable binding with no agent added.

## Methods

**Dispersed guinea pig pancreatic cell preparation.** Dispersed acini from guinea pig pancreas were prepared using the modification (48) of the method described previously (49).

**Amylase release.** Amylase release was determined as described previously (48, 49). The standard amylase release incubation solution contained 24.5 mM HEPES (pH 7.4), 98 mM NaCl, 6 mM KCl, 2.5 mM $NaH_2PO_4$, 5 mM sodium pyruvate, 5 mM sodium fumarate, 5 mM sodium glutamate, 2 mM glutamine, 11.5 mM glucose, 0.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5 mM theophylline, 0.01% (w/v) trypsin inhibitor, 1% (v/v) amino acid mixture, 1% (v/v) essential vitamin mixture, and 1% (w/v) BSA. Amylase activity was determined by using the Phadebas reagent (48, 49).

**Preparation of peptides.** [D-Tyr$^6$]Bn(6–13)ME was prepared as described previously (45), by treatment of the peptide resin with methanol containing 10% triethylamine. The crude peptide was purified on a column (2.5 × 90 cm) of Sephadex G-25, followed by elution with linear gradients of acetonitrile in 0.1% trifluoroacetic acid (flow rate, ~3 ml/min) on columns (1.5 × 50 cm) of Vydac $C_{18}$ silica (10–15 μM). Homogeneity of the peptide was assessed by analytical reverse phase HPLC; the peptide was >98% pure. Amino acid analysis gave the expected amino acid ratios.

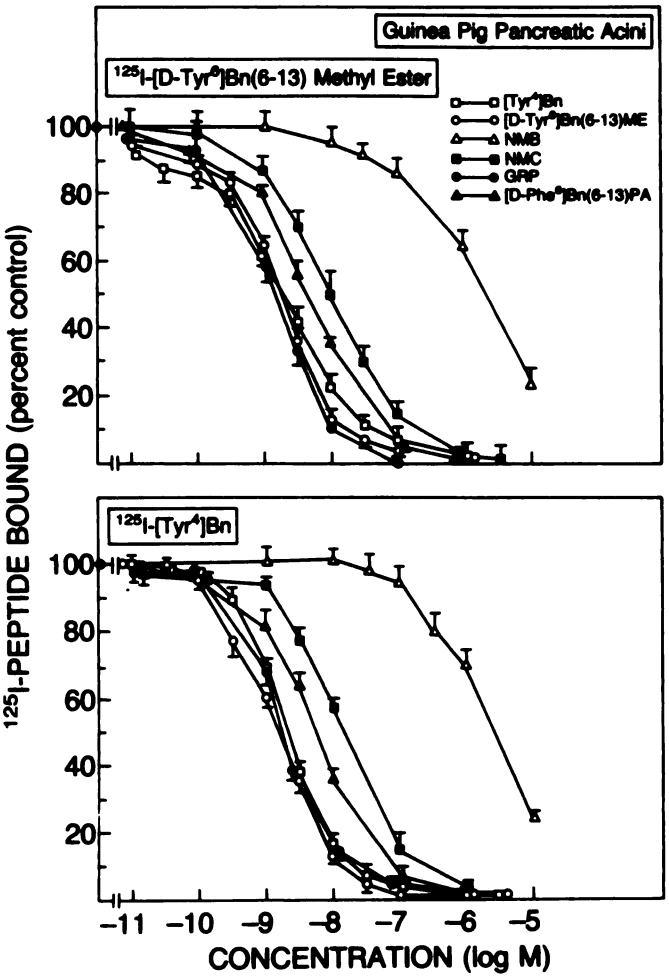


**Fig. 5.** Ability of various analogues of Bn to inhibit binding of the antagonist $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME (*top*) or the agonist $^{125}$I-[Tyr$^4$]Bn (*bottom*) to guinea pig pancreatic acini. Pancreatic acini were incubated with either 50 pM $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME (for 20 min) or 50 pM $^{125}$I-[Tyr$^4$]Bn (for 60 min), either alone, with 1 μM Bn, or with the indicated concentrations of the various Bn analogues, at 22°. Results are expressed as the percentage of saturable binding in the absence of unlabeled peptide. Values represent means of four separate experiments. *Vertical bar*, 1 SE. *PA*, propylamide.

**Preparation of $^{125}$I-[Tyr$^4$]Bn and $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME.** $^{125}$I-[Tyr$^4$]Bn (2200 Ci/mmol) was prepared using the modification (20, 21) of the method described previously (16). After iodination using Iodo-Gen, 1 M dithiotreitol was added and incubated at 80° for 60 min. After reverse phase HPLC on a column (0.46 × 25 cm) of μBondapak $C_{18}$, with isocratic elution with acetonitrile (22.5%) in TEAP (0.25 M, pH 3.5) at a flow rate of 1 ml/min, <7% of the radioactivity was incorporated in the oxidized peptide, which was separated by >3 min from the reduced iodinated peptide. $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME (2200 Ci/mmol) was prepared by dissolving 0.2 mg of Iodo-Gen in 10 ml of chloroform (0.02 μg/ml); 20 μl of this solution (0.4 μg of Iodo-Gen) were transferred to a vial, dried under a stream of nitrogen, and washed with 100 μl of $KH_2PO_4$ (pH 7.4). To this vial were added 30 μl of $KH_2PO_4$ (pH 7.4), 8 μg of [D-Tyr$^6$]Bn(6–13)ME in 4 μl of water, and 2 mCi of $Na^{125}I$; the solution was mixed and incubated at 24° for 6 min. The incubation was stopped by addition of 300 μl of water and the

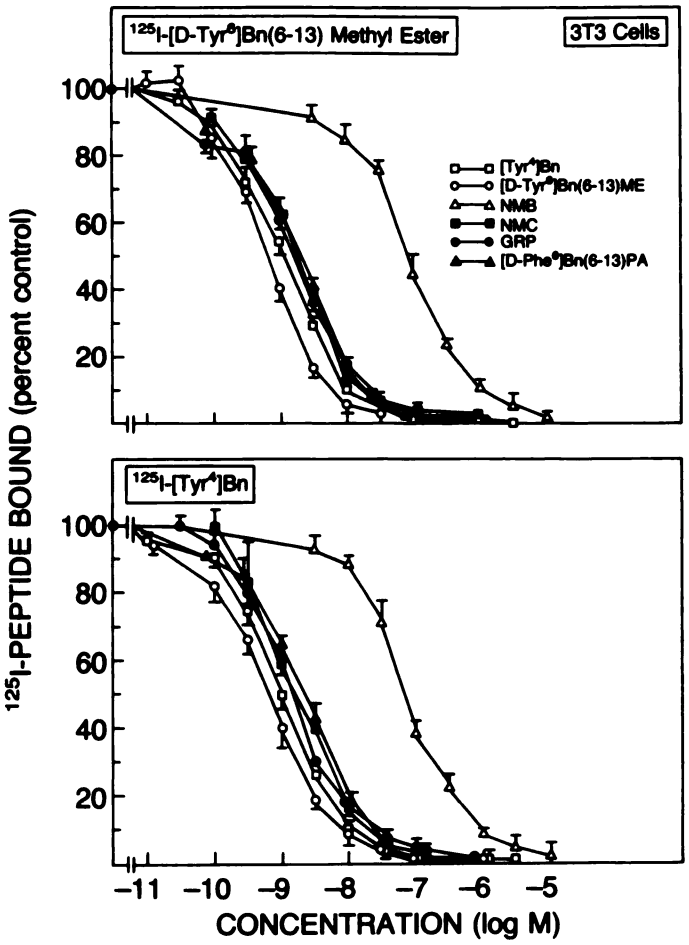


**Fig. 6.** Ability of various Bn analogues to inhibit binding of the antagonist $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME (*top*) or the agonist $^{125}$I-[Tyr$^4$]Bn (*bottom*) to 3T3 cells. Methods and data presentation are the same as outlined in the legend to Fig. 5.

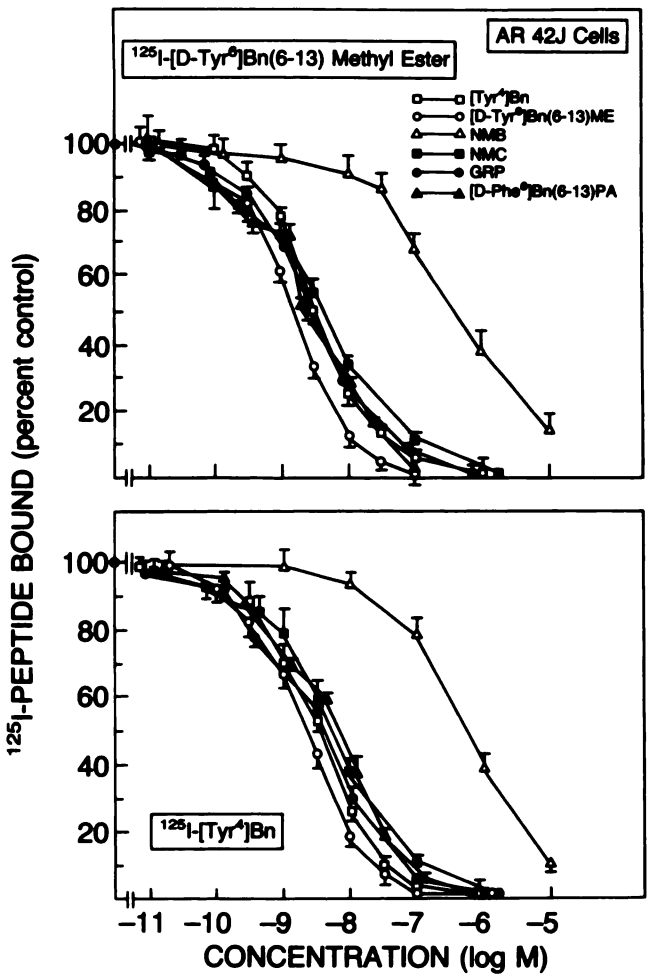


**Fig. 7.** Ability of various analogues of Bn to inhibit binding of the antagonist $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME (*top*) or the agonist $^{125}$I-[Tyr$^4$]Bn (*bottom*) to AR42J cells. Methods and data presentation are the same as outlined in the legend to Fig. 5.

incubated mixture was then loaded onto a SepPak cartridge. The SepPak cartridge was washed with 5 ml of 0.25 M TEAP and 5 ml of water, and the radiolabeled peptides were eluted with 50% (v/v) acetonitrile/0.25 M TEAP. $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME was purified using reverse phase HPLC and was eluted with 22.5% (v/v) acetronitrile/0.25 M TEAP. The pH was adjusted to 7.4 using 0.2 M Tris (pH 9.5), and the purified tracer was stored at −20°.

**Binding of $^{125}$I-[Tyr$^4$]Bn and $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME to pancreatic acini, 3T3 cells, or AR42J cells.** The standard binding buffer contained 50 mM HEPES (pH 7.4), 130 mM NaCl, 7.7 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.5% (w/v) BSA, 4 μg/ml leupeptin, 2 μg/ml chymostatin, and 0.02% (w/v) soybean trypsin inhibitor. Binding of $^{125}$I-[Tyr$^4$]Bn or $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME was performed using the method described previously for binding of $^{125}$I-[Tyr$^4$]Bn to various cells (45). Incubations contained 50 pM $^{125}$I-[Tyr$^4$]Bn or 50 pM $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME. Nonsaturable binding of $^{125}$I-[Tyr$^4$]Bn or $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME was the amount of radioactivity associated with the cells in incubations containing 50 pM ligand plus 1 μM Bn. Nonsaturable binding was <10% of total binding in all experiments.

**Growth of murine 3T3 cells and AR42J cells.** Stock cultures of 3T3 cells (kindly provided by Dr. E. Rozengurt, Imperial Cancer Research Fund, London, England) and AR42J cells (from the American Type Culture Collection, Rockville, MD) were grown in Dulbecco's modified Eagle medium supplemented with 10% bovine serum. The cells were grown in an atmosphere of 5% $CO_2$. On the day of the binding assay cells were suspended, by using a rubber policeman, in standard binding buffer at the indicated concentrations.

**Preparation of membranes from guinea pig or rat pancreas, rat esophagus, or 3T3 cells.** Homogenizing buffer contained 50 mM Tris (pH 7.4), 0.2 mg/ml soybean trypsin inhibitor, and 0.2 mg/ml benzamidine. Isolated rat esophagus, rat pancreas, or pancreas from male NIH-Hartley strain guinea pigs was minced with a pair of scissors (one pancreas or esophagus/5 ml of homogenizing buffer, 4°) and homogenized for 30 sec with a Polytron (Brinkman Instruments) at speed 6. The homogenized suspension was centrifuged at 1500 rpm for 10 min in a Sorvall RC-5B Superspeed centrifuge (DuPont). The supernatant was removed and recentrifuged at 20,000 rpm for 20 min. The pellet was resuspended in homogenizing buffer (one pancreas/20 ml or one esophagus/5 ml) and stored at −40°. 3T3 cell membranes were prepared in a similar manner and were stored at a concentration of 50 μg of protein/ml.

**Binding of $^{125}$I-[Tyr$^4$]Bn and $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME to cell membranes.** The standard membrane binding buffer contained 10 mM HEPES (pH 7.0), 118 mM NaCl, 4.7 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.2 mg/ml benzamidine, 0.2 mg/ml soybean trypsin inhibitor, and 0.2% (w/v) BSA. The binding assay contained 50 pM ligand and 15 μg of membrane protein from 3T3 cells or 150 μg of membrane protein from rat pancreas, rat esophagus, or guinea pig pancreas, with or without unlabeled peptides (final volume, 300 μl). At the times indicated, duplicate aliquots of 100 μl were taken, added to 5 ml of incubation buffer at 4°, filtered over GF/B glass filters (Whatman LabSales, Inc., Hillsboro, OR), and counted for bound radioactivity in a γ counter. Incubations were performed at 22° for 60 min with $^{125}$I-

TABLE 3

**Ability of various BN-related peptides to inhibit binding of the antagonist $^{125}$I-[D-Tyr$^6$]Bn(6-13)ME or the agonist $^{125}$I-[Tyr$^4$]Bn to dispersed guinea pig pancreatic acini, 3T3 cells, or AR42J cells**

Cell preparations were incubated with ligands as stated in the legends to Figs. 5–7. $K_d$ values (apparent dissociation constant) and $B_{max}$ (binding capacity) were determined from the data shown in Figs. 5–7, by using a nonlinear, least-squares, curve-fitting computer program (LIGAND) (53) and the method of Cheng and Prusoff (54). Values are means ± 1 SE from at least four separate experiments.

| Peptide[a] | $K_d$ | | | | | |
|---|---|---|---|---|---|---|
| | Guinea pig pancreatic acini | | 3T3 cells | | AR42J cells | |
| | $^{125}$I-ME | $^{125}$I-Bn | $^{125}$I-ME | $^{125}$I-Bn | $^{125}$I-ME | $^{125}$I-Bn |
| | nM | | | | | |
| Bn | 2.0 ± 0.2 | 1.8 ± 0.1 | 0.82 ± 0.09 | 0.90 ± 0.11 | 2.8 ± 0.2 | 2.2 ± 0.5 |
| GRP | 1.4 ± 0.1 | 1.7 ± 0.1 | 1.2 ± 0.1 | 1.4 ± 1.0 | 2.6 ± 0.2 | 3.7 ± 0.2 |
| GRP-18-27 | 7.8 ± 1.0 | 11.8 ± 0.9 | 1.3 ± 0.1 | 1.5 ± 0.1 | 3.5 ± 0.3 | 4.4 ± 0.2 |
| NB | 1574 ± 161 | 2889 ± 290 | 62 ± 6 | 60 ± 4 | 118 ± 8.7 | 287 ± 48 |
| [D-Tyr$^6$]Bn(6-13)ME | 1.4 ± 0.2 | 1.6 ± 0.1 | 0.53 ± 0.10 | 0.50 ± 0.1 | 1.1 ± 0.3 | 2.1 ± 0.1 |
| [D-Phe$^6$]Bn(6-13)PA | 4.6 ± 0.2 | 5.4 ± 0.6 | 1.2 ± 0.1 | 1.7 ± 0.1 | 2.7 ± 0.2 | 4.6 ± 6.5 |
| | $B_{max}$ | | | | | |
| | Guinea pig pancreatic acini | | 3T3 cells | | AR42J cells | |
| | $^{125}$I-ME | $^{125}$I-Bn | $^{125}$I-ME | $^{125}$I-Bn | $^{125}$I-ME | $^{125}$I-Bn |
| | pmol/mg of protein | | | | | |
| | 0.52 ± 0.1 | 0.73 ± 0.20 | 4.4 ± 0.4 | 11.3 ± 1.2[b] | 2.3 ± 0.4 | 4.6 ± 0.8[b] |

[a] PA, propylamide; $^{125}$I-ME, $^{125}$I-[D-Tyr$^6$]Bn(6-13)ME; $^{125}$I-Bn, $^{125}$I-[Tyr$^4$]Bn.
[b] Significantly different ($p < 0.05$) from the value obtained for the indicated cell type with the other ligand.

[Tyr$^4$]Bn and for 20 min with $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME unless otherwise indicated.

**Stripping of surface-bound $^{125}$I-[Tyr$^4$]Bn and $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME.** After incubation with 50 pM $^{125}$I-[Tyr$^4$]Bn or 50 pM $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME for the indicated times and at the indicated temperatures, samples (100 μl) were added to 0.2 M acetic acid with 0.5 M NaCl (pH 2.5) (acid wash), 0.2 M glycine with 0.5 M NaCl (pH adjusted to 10.5 with 1 N NaOH) (base wash), 0.5 M potassium thiocyanate (KSCN wash), or standard incubation buffer (control) for 5 min at 4° to remove surface-bound radioligand, as described previously (50–52).

**Statistical significance.** Experiments involving curve fitting were performed in a paired fashion and the differences were compared using the Student's $t$ test; a value of $p < 0.05$ was considered significant.

## Results

### Amylase Release

The Bn-related agonists Bn, GRP, GRP-18–27 (NMC), and NMB caused a 7-fold stimulation of enzyme secretion from dispersed guinea pig pancreatic acini (Fig. 1, *left*). Bn was the most potent, causing half-maximal stimulation at 0.3 nM. GRP was 2-fold less potent ($EC_{50}$, 0.6 nM), GRP-18–27 4-fold less potent ($EC_{50}$, 1.2 nM), and NMB 300-fold less potent ($EC_{50}$, 100 nM). Neither [D-Tyr$^6$]Bn(6–13)ME nor the reported Bn receptor antagonist [D-Phe$^6$]Bn(6–13)propylamide stimulated enzyme secretion at concentrations as high as 10 μM. In contrast, each of these peptides caused a dose-dependent decrease in Bn-stimulated amylase release (Fig. 1, *right*). Each peptide was equipotent, causing half-maximal inhibition at 1.6 ± 0.3 nM.

### Binding

**Kinetics.** The time course and temperature dependence of binding of the radiolabeled agonist $^{125}$I-[Tyr$^4$]Bn and the radiolabeled antagonist $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME were compared using Bn receptors in guinea pig pancreatic acini (Fig. 2, *left*) and in two cell lines with well described Bn receptors, i.e., murine 3T3 cells (Fig. 2, *middle*) and the rat pancreatic tumor cell line AR42J (Fig. 2, *right*) (10, 13). With each cell line, the binding of both ligands was saturable, temperature dependent, and time dependent, but the two ligands differed in their kinetics. Specifically, with guinea pig pancreatic acini (Fig. 2) half-maximal and maximal binding of $^{125}$I-[Tyr$^4$]Bn at 37° or 22° was reached at 20 min and 80 min, respectively, and binding at 4° was slower, reaching only 25% of the amount seen at 37° or 22° by 100 min. Addition of 1 μM Bn decreased binding at 37° or 22° by 90%. In contrast, binding of the radiolabeled antagonist $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME was much more rapid at all temperatures, reaching maximal levels at 5 min (Fig. 2) at 37°. Kinetics, saturability, and time and temperature dependence similar to those seen in guinea pig pancreatic acini were seen for both ligands with Bn receptors on murine 3T3 cells (Fig. 2, *middle*) and AR42J cells (Fig. 2, *right*).

To investigate further the kinetics of binding, the reversibility of binding of the two ligands was compared (Fig. 3). For guinea pig pancreatic acini at 37° dissociation of the radiolabeled antagonist was rapid, with half-maximal dissociation at 5 min. Lowering the temperature to 4° slowed the dissociation such that only 20% dissociated by 30 min. In contrast, the dissociation rate of the radiolabeled agonist $^{125}$I-[Tyr$^4$]Bn was much slower at 37° (Fig. 3, *top right*), in that only 20% of bound radiolabeled $^{125}$I-[Tyr$^4$]Bn dissociated by 30 min; at 4° only 10% dissociated by 60 min (Fig. 3, *top right*). Similar results were obtained with murine 3T3 cells (Fig. 3, *middle*) and AR42J cells (Fig. 3, *bottom*).

**Surface-bound ligand stripping.** To investigate the possibility that the difference in kinetics might be due to differences in internalization of the two ligands by the different cells, ligand-stripping experiments to remove surface-bound radioligand were done for each ligand with 3T3 cells (Fig. 4; Table 1) and guinea pig pancreatic acini or AR42J cells (Table 1). With 3T3 cells, an acid, base, or potassium thiocyanate wash removed 98 ± 1%, 43 ± 5%, and 30 ± 4%, respectively, of the antagonist bound during a previous incubation for 10 min at 37° and 87 ± 3%, 35 ± 3%, and 20 ± 2% of the antagonist bound during a

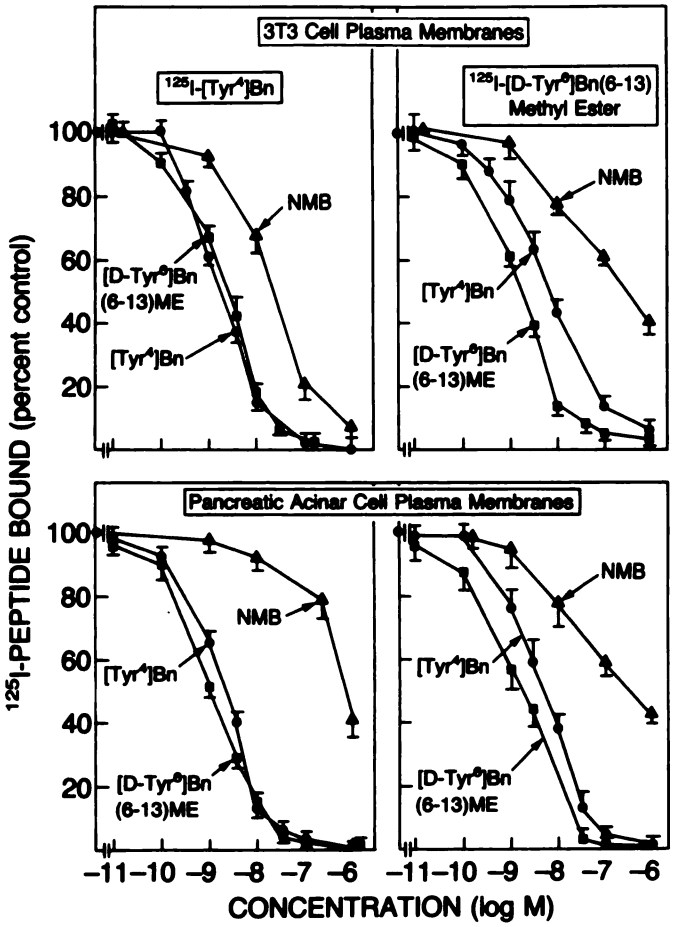


**Fig. 8.** Ability of various Bn-related peptides to inhibit binding of the antagonist $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME (*right*) or the agonist $^{125}$I-[Tyr$^4$]Bn (*left*) to cell membranes from Swiss 3T3 cells (*top*) and from guinea pig pancreas (*bottom*). Cell membranes from guinea pig pancreas and from 3T3 cells were prepared as described in Experimental Procedures. Cell membranes were incubated with 50 pM $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME (for 30 min) or 50 pM $^{125}$I-[Tyr$^4$]Bn (for 60 min), either alone, with 1 μM Bn, or with the indicated concentrations of the various Bn analogues, at 22°. Results are expressed as the percentage of saturable binding in the absence of unlabeled peptide. Values represent means of at least four separate experiments. *Vertical bar*, 1 SE.

TABLE 4

**Results of acid stripping of antagonist $^{125}$I-[D-Tyr$^6$]Bn(6-13)ME or agonist $^{125}$I-[Tyr$^4$]Bn bound to 3T3 cell or guinea pig pancreatic acinar cell membranes**

Cell membranes were incubated with 50 pM $^{125}$I-[D-Tyr$^6$]Bn(6-13)ME or 50 pM $^{125}$I-[Tyr$^4$]Bn for 40 min at 37° and acid strippability was determined using 0.2 M acetic acid and 0.5 M NaCl (pH 2.5), as described in Experimental Procedures. Results are expressed as the percentage of control (incubation with buffer) saturable binding remaining after acid treatment. Data represent means ± 1 SE from at least three separate experiments.

| Cell membranes from | $^{125}$I-Peptide bound | |
|---|---|---|
| | $^{125}$I-[D-Tyr$^6$]Bn(6-13)ME | $^{125}$I-[Tyr$^4$]Bn |
| | % acid resistant | |
| 3T3 cells | 0.6 ± 0.2 | 0.5 ± 0.2 |
| Guinea pig pancreatic acini | 7.7 ± 3.5 | 6.0 ± 2.2 |

previous incubation of the 3T3 cells for 60 min at 37° (Fig. 4, *top*; Table 1). In contrast, after a previous 10-min incubation of 3T3 cells with the agonist $^{125}$I-[Tyr$^4$]Bn an acid wash, base wash, or potassium thiocyanate wash removed 36 ± 3, 30 ± 5, and 20 ± 3%, respectively, of the bound ligand, whereas after a 60-min previous incubation an acid wash, base wash, or potassium thiocyanate wash removed 17 ± 2, 10 ± 3, and 8 ± 2% of the bound ligand. These results show that in 3T3 cells the radiolabeled agonist is increasingly not acid strippable and is likely internalized with time, whereas the bound radiolabeled antagonist is minimally, if at all, internalized in 3T3 cells. In AR42J cells and guinea pig pancreatic acini (Table 1) a similar pattern was observed; however, the extent of internalization differed. In AR42J cells after a 60-min incubation with the agonist $^{125}$I-[Tyr$^4$]Bn 36% of the bound ligand was not acid strippable; in guinea pig pancreatic acini the value was 23% (Table 1). In contrast, with the antagonist in AR42J cells or guinea pig pancreatic acini >90% of the bound ligand was acid strippable at all times (Table 1).

**Affinity and specificity.** In each of the three cell systems, the binding of $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME was inhibited by Bn receptor agonists (Bn, GRP, GRP-18–27, or NMB) or Bn receptor antagonists [[D-Phe$^6$]Bn(6–13)propylamide or [D-Tyr$^6$]Bn(6–13)ME] but not by agents that interact with other receptors (Table 2). Similar results were obtained with binding of the radiolabeled agonist $^{125}$I-[Tyr$^4$]Bn to each cell system (data not shown).

To compare the relative affinities of various Bn receptor agonists and antagonists for receptors on guinea pig pancreatic acini (Fig. 5), 3T3 cells (Fig. 6), or AR42J cells (Fig. 7) determined by using a radiolabeled agonist or the radiolabeled antagonist, we compared dose-inhibition curves for each ligand with each peptide. With guinea pig pancreatic acini the agonists Bn and GRP and the antagonist [D-Tyr$^6$]Bn(6–13)ME were equipotent at inhibiting binding of $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME, with each causing half-maximal inhibition at 1.5 nM (Fig. 5, *top*; Table 3). The antagonist [D-Phe$^6$]Bn(6–13)propylamide was 3-fold less potent and the agonists GRP-18–27 and NMB were 5- and 1120-fold less potent, respectively. Similar relative and absolute values were obtained when dose-inhibition curves for each unlabeled agonist and antagonist were determined using the radiolabeled agonist as the radiolabeled ligand (Fig. 5, *bottom*; Table 3). Similar studies were done with 3T3 cells and AR42J cells, which had demonstrated different degrees of internalization of the radiolabeled agonist $^{125}$I-[Tyr$^4$]Bn. With both 3T3 cells (Fig. 6; Table 3) and AR42J cells (Fig. 7; Table 3) similar results were obtained with both ligands for both the relative and absolute affinities of the different agonists and antagonists.

To investigate further the ability of each ligand to characterize the Bn receptors on each cell type, the dose-inhibition curves were analyzed using a least-squares curve-fitting program (LIGAND) (53). For each cell type the dose-inhibition curves were best fit by a single-binding site model (Table 3). However, the results differed in the number of binding sites per cell with the different cell systems, as determined with the two ligands (Table 3). In guinea pig pancreatic acini the numbers of binding sites were not significantly different with the two ligands; however, with 3T3 cells and AR42J cells 2.6 and 2 times, respectively, as many binding sites were obtained using the radiolabeled agonist, compared with the radiolabeled antagonist (Table 3).

**Kinetics and stoichiometry in membranes.** To determine further whether the differences in the numbers of binding sites obtained in the different cells using the radiolabeled agonist or the radiolabeled antagonist might be due to the

TABLE 5

**Ability of various Bn-related peptides to inhibit binding of the agonist $^{125}$I-[Tyr$^4$]Bn or the antagonist $^{125}$I-[D-Tyr$^6$]Bn(6-13)ME to cell membranes from guinea pig pancreas or Swiss 3T3 cells**

Cell membranes from guinea pig pancreas and Swiss 3T3 cells were prepared as stated in Experimental Procedures. Cell membranes were incubated with 50 pM levels of each ligand and various concentrations of unlabeled peptide as described in the legend to Fig. 8. $K_d$ values (apparent dissociation constant) and $B_{max}$ (binding capacity) were determined from the data shown in Fig. 8 by using a nonlinear, least-squares, curve-fitting computer program (LIGAND) (53) and the method of Cheng and Prusoff (54). Values are means ± 1 SE from at least three separate experiments.

| Peptide | $K_d$ | | | |
|---|---|---|---|---|
| | Guinea pig pancreas membranes | | 3T3 cell membranes | |
| | $^{125}$I-[D-Tyr$^6$]Bn(6-13)ME | $^{125}$I-[Tyr$^4$]Bn | $^{125}$I-[D-Tyr$^6$]Bn(6-13)ME | $^{125}$I-[Tyr$^4$]Bn |
| | nM | | | |
| [Tyr$^4$]Bn | 3.1 ± 0.4 | 2.0 ± 0.2 | 4.3 ± 0.6 | 1.6 ± 0.1 |
| NMB | 137 ± 21 | 473 ± 51 | 151 ± 3.0 | 255 ± 37 |
| [D-Tyr$^6$]Bn(6-13)ME | 1.9 ± 0.3 | 1.1 ± 0.1 | 1.7 ± 0.1 | 2.0 ± 0.2 |
| | $B_{max}$ | | | |
| | Guinea pig pancreas membranes | | 3T3 cell membranes | |
| | $^{125}$I-[D-Tyr$^6$]Bn(6-13)ME | $^{125}$I-[Tyr$^4$]Bn | $^{125}$I-[D-Tyr$^6$]Bn(6-13)ME | $^{125}$I-[Tyr$^4$]Bn |
| | pmol/mg of protein | | | |
| | 0.07 ± 0.02 | 0.08 ± 0.01 | 1.3 ± 0.2 | 2.2 ± 0.4 |

differences in internalization of the two ligands, binding studies with both ligands were done using membranes from guinea pig pancreatic acini and 3T3 cells (Fig. 8). With membranes from both cell types with both ligands, >93% of the bound ligand was acid strippable (Table 4).

Both the Bn receptor agonists [Tyr$^4$]Bn and NMB and the Bn receptor antagonist [D-Tyr$^6$]Bn(6–13)ME inhibited binding of both ligands to 3T3 or guinea pig pancreatic acinar cell membranes in a concentration-dependent manner (Fig. 8). For membranes from both cell types with both ligands, Bn and [D-Tyr$^6$]Bn(6–13)ME were approximately equipotent, causing half-maximal inhibition at 1.3 nM (Fig. 8). NMB was 100–200-fold less potent for each ligand (Fig. 8; Table 5). When the ability of Bn to inhibit binding of the radiolabeled agonist and of the antagonist [D-Tyr$^6$]Bn(6–13)ME to inhibit binding of the radiolabeled antagonist were analyzed using a nonlinear least-squares curve-fitting program (LIGAND) (53), for both ligands in each cell type the dose-inhibition curves were best fit by a single-binding site model (Table 5). However, in contrast to the findings with cells, no significant differences were found in the binding capacities of the cell membrane preparations for the agonist or the antagonist (compare Tables 3 and 5).

**Effect of guanine nucleotides in membranes.** To investigate the effects of activation of a guanine nucleotide-binding protein on the binding of $^{125}$I-[Tyr$^4$]Bn or $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME to the Bn receptor, the effect of increasing concentrations of the nonhydrolyzable guanosine analogue Gpp(NH)p on binding of each ligand to 3T3 or guinea pig pancreatic acinar cell membranes was determined (Fig. 9). Gpp(NH)p caused a concentration-dependent decrease in binding of the agonist $^{125}$I-[Tyr$^4$]Bn to 3T3 (Fig. 9, *top*) and guinea pig pancreatic acinar (Fig. 9, *bottom*) cell membranes. With 3T3 cell membranes half-maximal inhibition of $^{125}$I-[Tyr$^4$]Bn binding occurred with 0.1 μM Gpp(NH)p, and with guinea pig pancreatic acinar cell membranes half-maximal inhibition occurred with 0.2 μM Gpp(NH)p. Maximal inhibition of binding of the agonist $^{125}$I-[Tyr$^4$]Bn (70–80%) occurred with 0.1–1 mM Gpp(NH)p with both cell membranes. No effect on binding of the antagonist $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME to cell membranes of either tissue was seen with concentrations up to 0.1 mM Gpp(NH)p (Fig. 9). To investigate further the effects of guanine nucleotides, the effect of a fixed concentration of Gpp(NH)p on the dose-inhibition curve for Bn inhibition of binding of the agonist $^{125}$I-[Tyr$^4$]Bn was determined in each tissue (Fig. 10). In 3T3 cell membranes 0.1 μM Gpp(NH)p and in guinea pig pancreatic acinar cell membranes 0.2 μM Gpp(NH)p decreased binding of the agonist $^{125}$I-[Tyr$^4$]Bn by 40–50% (Fig. 10). Gpp(NH)p caused a significant decrease in the affinity of Bn for the Bn receptors in both guinea pig pancreatic acinar cell membranes and 3T3 cell membranes, with no change in receptor number in either cell type (Table 6).

**Distinguishing Bn receptor subtypes.** Recently, various Bn receptor subtypes have been proposed (5, 22–26). To determine whether the two radioligands differ in their abilities to distinguish these proposed Bn receptor subtypes, the abilities of each labeled ligand to bind to rat pancreas (Fig. 11, *top*) and esophageal membranes (Fig. 11, *bottom*), which are known to contain the different Bn receptor subtypes (22, 26), were compared. Binding of both radiolabeled antagonist and agonist to rat pancreatic acinar cell membranes was time dependent, and addition of 1 μM Bn decreased binding by 85% (Fig. 11, *top*). In contrast, rat esophageal membranes bound the agonist $^{125}$I-[Tyr$^4$]Bn in a fashion similar to that of pancreatic membranes, whereas no saturable binding of the antagonist $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME occurred (Fig. 11, *bottom*). To investigate further the basis for this, the affinities of Bn receptors on rat esophageal or pancreatic acinar cell membranes for the agonist Bn and the antagonist [D-Tyr$^6$]Bn(6–13)ME were compared (Fig. 12). Similarly to guinea pig pancreas, the Bn receptor on rat pancreatic acinar cell membranes had a high affinity for both the agonist Bn and the antagonist [D-Tyr$^6$]Bn(6–13)ME (compare Figs. 8 and 12). With either the radiolabeled agonist or the radiolabeled antagonist, half-maximal inhibition occurred at 1–2 nM (Fig. 12, *bottom*), demonstrating that this Bn receptor had a high affinity for both ligands. In rat esophagus, Bn was also potent at inhibiting binding of the radiolabeled agonist $^{125}$I-[Tyr$^4$]Bn, causing half-maximal at 2 nM (Fig. 12, *top*). In contrast, the antagonist [D-Tyr$^6$]Bn(6–13)ME had a >10,000-fold lower affinity, causing no inhibition at concentrations up

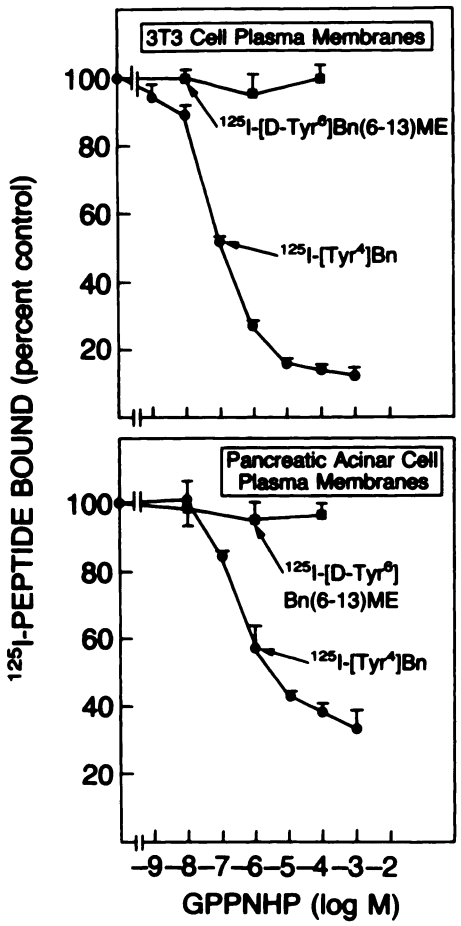


**Fig. 9.** Effect of the guanine nucleotide analogue Gpp(NH)p on the binding of the radiolabeled antagonist $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME or the radiolabeled agonist $^{125}$I-[Tyr$^4$]Bn to Swiss 3T3 cell or pancreatic acinar cell membranes. 3T3 cell (*top*) or pancreatic acinar cell (*bottom*) membranes were incubated at 22° with either 50 pM $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME (for 20 min) or 50 pM $^{125}$I-[Tyr$^4$]Bn (for 60 min), either alone, with 1 μM unlabeled Bn, or with the indicated concentrations of Gpp(NH)p. Results are expressed as the percentage of saturable binding in the absence of unlabeled peptide. Values represent means of at least four separate experiments. *Vertical bar*, 1 SE.

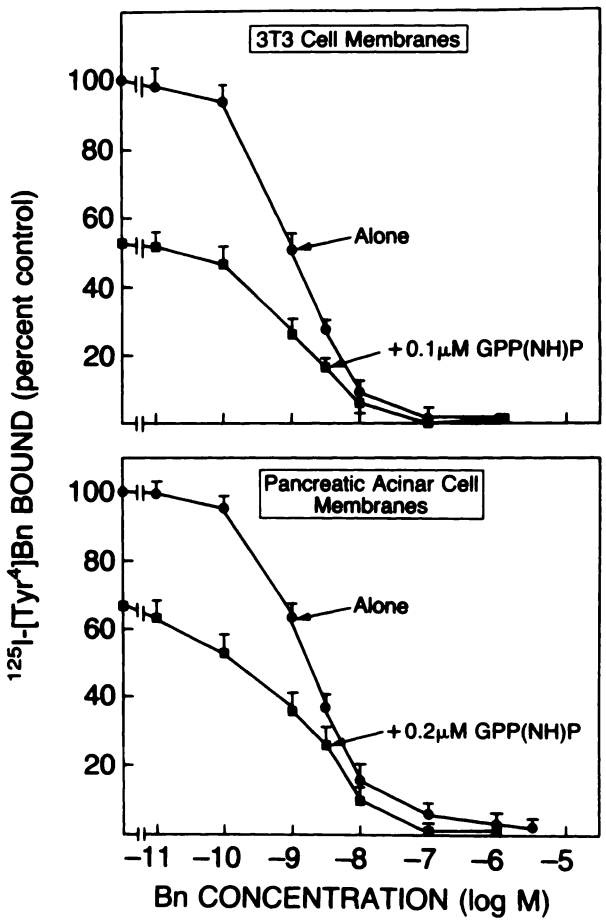


**Fig. 10.** Effect of the guanine nucleotide Gpp(NH)p on the Bn dose-inhibition curve for binding of the radiolabeled agonist $^{125}$I-[Tyr$^4$]Bn to 3T3 cell (*top*) or guinea pig pancreatic acinar cell (*bottom*) membranes. Cell membranes were incubated at 22° either with or without 0.1 μM Gpp(NH)p and with 50 pM $^{125}$I-[Tyr$^4$]Bn, in the presence of the indicated concentrations of unlabeled peptide. Results are expressed as the percentage of saturable binding in the absence of unlabeled peptide with no Gpp(NH)p present. Values represent means of at least four separate experiments. *Vertical bar*, 1 SE.

to 1 μM (Fig. 12, *top*), demonstrating that this subtype of Bn receptor had a low affinity for the antagonist [D-Tyr$^6$]Bn(6–13)ME.

## Discussion

All of the information available from ligand-binding studies characterizing receptors for most gastrointestinal peptides, such as Bn, secretin, vasoactive intestinal peptide, substance P-related peptides, and calcitonin gene-related peptide-like peptides, have come from studies using radiolabeled agonists. Numerous studies of agents that interact with receptors for GnRH, angiotensin, cholinergic agents, or CCK or adrenergic receptors have demonstrated that there are advantages to using both radiolabeled antagonists and radiolabeled agonists to characterize these receptors (34–43, 55–57). Radiolabeled antagonists have the advantage that they often distinguish subtypes of receptors (35, 55, 56). In addition, agonists may induce different affinity states, which can make it more difficult to determine total receptor number (34–38). The agonist receptor affinity may be influenced by guanine nucleotides, and the affinity may be affected by the presence of various ions such as $Na^+$ or $Mg^{2+}$ (34–38, 55–57). Agonists may be internalized, making it difficult to obtain an accurate characterization of cell surface receptor affinity or number (39–41, 51, 52, 58, 59). Furthermore, when a significant percentage of the radiolabeled ligand is internalized and not dissociable conventional analysis of receptor number or affinity may be limited, because the

TABLE 6

**Effect of Gpp(NH)p on affinity and binding capacity determined from binding of the agonist $^{125}$I-[Tyr$^4$]Bn to cell membranes from guinea pig pancreatic acini and 3T3 cells**

$K_d$ (apparent dissociation constant) and $B_{max}$ (binding capacity) were determined from the data shown in Fig. 10 using a nonlinear, least-squares, curve-fitting computer program (LIGAND) (53). Values are means ± 1 SE from four separate experiments.

| Gpp(NH)p | Pancreatic acinar cell membranes | | 3T3 cell membranes | |
|---|---|---|---|---|
| | $K_d$ | $B_{max}$ | $K_d$ | $B_{max}$ |
| μM | nM | pmol/mg of protein | nM | pmol/mg of protein |
| 0 | 2.0 ± 0.2 | 0.08 ± 0.02 | 1.7 ± 0.2 | 2.2 ± 0.1 |
| 0.1 | | | 2.9 ± 0.2[a] | 1.9 ± 0.1 |
| 0.2 | 3.0 ± 0.05[a] | 0.07 ± 0.01 | | |

[a] $p < 0.01$, compared with value with no Gpp(NH)p present.

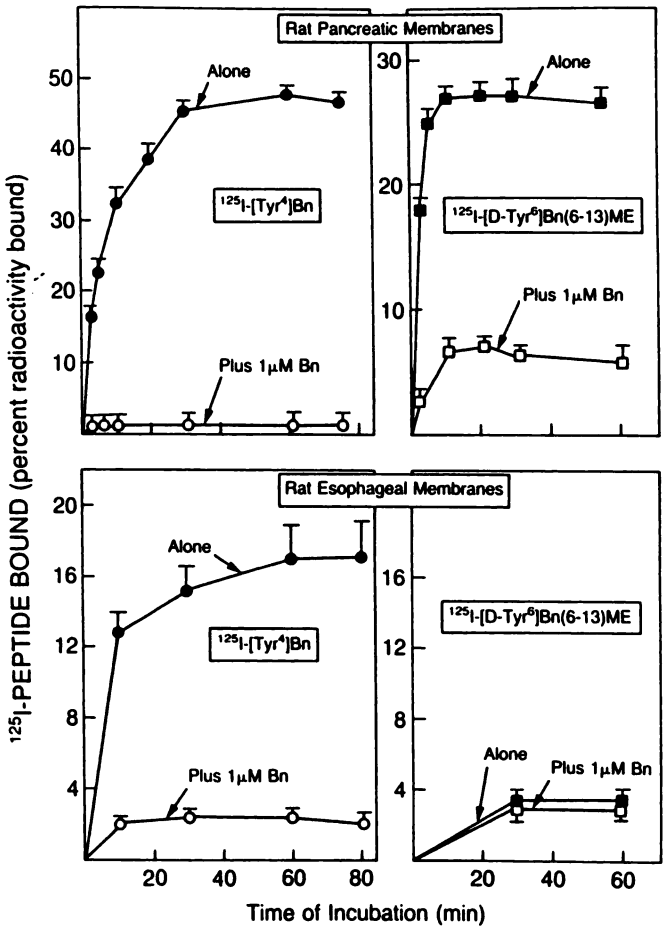


**Fig. 11.** Time course of binding of the radiolabeled agonist $^{125}$I-[Tyr$^4$]Bn or the antagonist $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME to rat pancreatic (*top*) or esophageal (*bottom*) cell membranes. Rat pancreatic or esophageal membranes were incubated with 50 pM $^{125}$I-[Tyr$^4$]Bn alone (●) or in the presence of 1 μM Bn (○) (*left*) or with 50 pM $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME alone (■) or in the presence of 1 μM Bn (□) (*right*), at 22°, for the indicated times. Results are expressed as the percentage of the added counts bound. In each experiment each value was determined in duplicate and results are means of at least three separate experiments. *Vertical bar*, 1 SE.

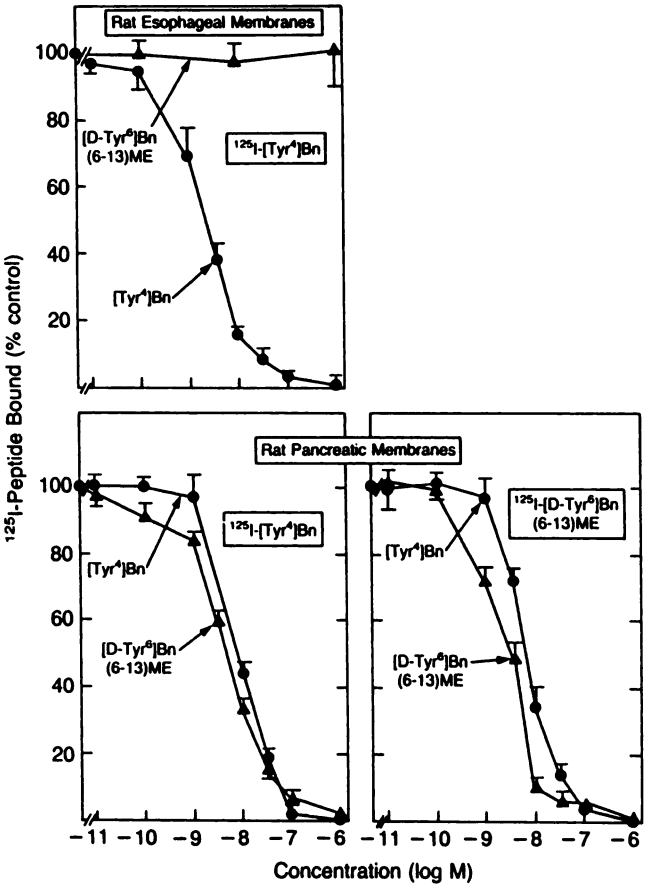


**Fig. 12.** Ability of the agonist [Tyr$^4$]Bn or the antagonist [D-Tyr$^6$]Bn(6–13)ME to inhibit binding of $^{125}$I-[Tyr$^4$]Bn or $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME to rat esophageal or pancreatic acinar cell membranes. Cell membranes from rat pancreas (*bottom*) were incubated at 22° for 20 min with 50 pM $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME (*right*) or for 60 min with 50 pM $^{125}$I-[Tyr$^4$]Bn (*left*), either alone or with the indicated concentrations of unlabeled peptides. Membranes from rat esophagus (*top*) were incubated at 22° for 60 min with 50 pM $^{125}$I-[Tyr$^4$]Bn, either alone or with the indicated peptides. Results are expressed as the percentage of the saturable binding in the absence of unlabeled peptide. Values represent means of three separate experiments. *Vertical bar*, 1 SE.

conditions required for Scatchard analysis are not met (39–41, 51, 52, 58, 59). Lastly, agonists for a number of different receptors have been reported to cause desensitization, which may lead to changes in receptor number or affinity, whereas antagonists do not cause desensitization (58–63).

High affinity receptors for Bn-related peptides have been characterized by ligand-binding studies in pancreatic acinar tissue (7, 16), CNS (18, 19), gastric antral cells (17), gastrointestinal smooth muscle (17), Swiss 3T3 cells (10), and various tumor cell lines (8, 12–16). In each case characterization has been done using radiolabeled agonists such as radiolabeled [Tyr$^4$]Bn, NMB, or GRP, because no high affinity radiolabeled antagonists had been described. In the present studies we provide evidence that $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME is a specific, high affinity, radiolabeled antagonist that recognizes Bn receptors in three different cell systems with well characterized Bn receptors and thus should be generally useful for the study of Bn receptors. The evidence that [D-Tyr$^6$]Bn(6–13)ME was a Bn receptor antagonist was that, similarly to the analogues [D-Phe$^6$]Bn(6–13)ethyl ester and [D-Phe$^6$]Bn(6–13)ME described in a previous study (45), [D-Tyr$^6$]Bn(6–13)ME did not have any agonist activity at the Bn receptors in guinea pig pancreas acini at concentrations as high as 10 μM; however, it caused a dose-dependent inhibition of Bn-stimulated enzyme secretion. The fact that the radiolabeled antagonist was binding to a Bn receptor was supported by the results that demonstrated that binding was rapid, time and temperature dependent, reversible, and saturable in each of the three different cell systems examined. The fact that this radiolabeled analogue was a specific Bn recepter ligand was demonstrated by the finding that only Bn or agents that interact with Bn receptors, such as the Bn receptor agonists Bn, GRP, GRP-18–27, or NMB (1, 7) or the Bn receptor antagonists [D-Tyr$^6$]Bn(6–13)ME or [D-Phe$^6$]Bn(6–13)propylamide (20, 45), inhibited the binding of the radiolabeled antagonist to 3T3 cells, AR42J cells, or guinea pig pancreatic acini, whereas agents that interact with other receptors, such as substance P, muscarinic cholinergic agents, gastrin, CCK, calcitonin gene-related peptide, or vasoactive intestinal peptide (64), had no effect. The high affinity of the radiolabeled antagonist was demonstrated by the finding that in each of the three different cell systems its affinity for the

Bn receptor was 0.5–1.5 nM, which was similar to the affinity of these receptors for Bn itself. The fact that the radiolabeled antagonist was binding to the Bn receptors that altered cell function was supported by the results showing that in each cell system the relative potencies of the various Bn receptor agonists and antagonists obtained from ligand studies using the radiolabeled antagonist $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME agreed closely with those obtained using the radiolabeled agonist $^{125}$I-[Tyr$^4$]Bn. Furthermore, in guinea pig pancreatic acini there was a close association between the relative abilities of the Bn receptor agonists to stimulate or of the antagonists [D-Tyr$^6$]Bn(6–13)ME or [D-Phe$^6$]Bn(6–13)propylamide to inhibit Bn-stimulated enzyme secretion and their abilities to occupy the Bn receptor. There was also a close relative correlation between the affinities of the various Bn receptor agonists obtained from the binding studies with the antagonist $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME in 3T3 cells and their previously reported abilities to alter [$^3$H]thymidine incorporation in these cells (45). Each of the latter observations demonstrates a close correlation between the binding affinities assessed using the radiolabeled antagonist $^{125}$I-[D-Tyr$^6$]Bn(6–13)ME and the abilities of the peptides to alter biological activity, suggesting that binding is reflective of Bn receptor occupation.

Similarly to studies comparing the interaction of radiolabeled agonists and radiolabeled antagonists with receptors for CCK/gastrin peptides, adrenergic agents, muscarinic cholinergic agents, GnRH, and angiotensin II on various cells (34–43, 55–57), in the present study the interactions of the radiolabeled Bn receptor agonist and the radiolabeled Bn receptor antagonist demonstrated similarities and some important differences. The two ligands gave similar results in assessing the affinity of agonists or antagonists for Bn receptors in each of the three different cell systems. The findings that similar receptor affinities of antagonists were determined from the binding of a radiolabeled agonist or antagonist are similar to findings reported for CCK receptors, gastrin receptors, and $\alpha$- or $\beta$-adrenergic receptors (37, 38, 55–57) but different from results with LHRH receptors, for which different affinities were obtained with the two types of ligands (42). The finding that similar affinities for agonists were determined from binding of the radiolabeled Bn receptor agonist or antagonist is similar to results of studies with angiotensin receptors (36, 42) but different from the results of studies with receptors for CCK, gastrin, adrenergic agents, and LHRH (37, 38, 51–55). With the LHRH receptor higher affinities for agonists were obtained from studies with a radiolabeled antagonist, compared with radiolabeled agonists (42), whereas with CCK, gastrin, and adrenergic receptors the reverse was true (37, 38, 55–57).

Similarly to receptors for a number of other agents (34–43, 55–57, 65, 66), the radiolabeled Bn receptor antagonist and agonist differed in their kinetics of binding, their extent of internalization, their assessment of the maximal binding capacity, the effect of guanine nucleotides on the binding of each, and their ability to distinguish receptor subtypes. The binding of the radiolabeled antagonist in each cell system more rapidly reached equilibrium and was more rapidly reversible than that of the radiolabeled agonist. This difference in kinetics of binding was probably secondary, at least to some extent, to the internalization of the radiolabeled Bn agonist and not the antagonist. Radiolabeled Bn-related agonists were reported previously to be internalized in 3T3 cells and the insulin-secreting pancreatic tumor cell line HIT-T15 (14, 15, 27, 28). The present study confirms these results in 3T3 cells and extends them by showing differing extents of internalization in guinea pig pancreatic acinar cells and the rat pancreatic tumor cell line AR42J. In the present study the fact that the radiolabeled Bn receptor agonist was internalized was shown by its failure to be removed by acid stripping and further supported by the fact that the radiolabeled Bn receptor agonist and radiolabeled antagonist were both completely acid-strippable from the membranes. In contrast to the radiolabeled Bn receptor agonist, in each cell system the radiolabeled Bn receptor antagonist was not internalized and dissociated rapidly. Similar results have been demonstrated for GnRH receptors by some (67) but not by others (39–41, 68, 69). Another difference in the binding results with the agonist and radiolabeled Bn antagonist was the assessment of the maximal number of Bn receptors per cell. At least two results suggest that this was due to the internalization of the radiolabeled Bn receptor agonist. In 3T3 cells and AR42J cells, which had the highest level of internalization of radiolabeled Bn agonist, the maximal binding capacity of the cells was significantly greater in studies with radiolabeled Bn agonist than in studies with the radiolabeled Bn antagonist, whereas in guinea pig pancreatic acini, which had the lowest level of internalization of the radiolabeled agonist, the binding capacities between the two ligands did not differ. In addition, in contrast to cells, equal numbers of binding sites were seen with both ligands in membranes from 3T3 cells or pancreatic acinar cells. It is unknown whether in the various cells the Bn receptor is, in fact, internalized or whether this difference is due entirely to radiolabeled agonist in intracellular organelles; however, this result demonstrates that an accurate assessment of cell surface receptors can only be obtained using the radiolabeled Bn antagonist.

In previous studies the binding of radiolabeled Bn agonists to Bn receptors on 3T3 cells and the insulinoma cell line HIT-T15 has been shown to be affected by guanine nucleotides (28–30). A similar finding was obtained in the present study for each of the three different cell types possessing Bn receptors, in that in each the nonhydrolyzable guanine nucleotide Gpp(NH)p decreased the binding of the radiolabeled agonist, due to a decrease in affinity of the Bn receptor for the radiolabeled agonist. In contrast, guanine nucleotides had no effect on the binding of the radiolabeled antagonist. Similar results for the differential effects of guanine nucleotides in regulating radiolabeled agonist and antagonist receptor interaction have been shown for a number of different receptors, such as those for $\alpha$- and $\beta$-adrenergic agents, CCK, gastrin, and angiotensin II (36–38, 55, 56). These results have possible significance in a number of areas. They raise the possibility that Bn receptor agonists and antagonists could be distinguished by the ability of Gpp(NH)p to affect their binding. In a number of recent studies (70, 71), attempts at purifying the Bn receptor have been reported using radiolabeled Bn agonist receptor assays. Because solubilized receptors may lose considerable affinity for radiolabeled agonists (38, 72), perhaps secondary to loss of association with a guanine nucleotide-binding protein, the lack of effect of Gpp(NH)p on the binding of the radiolabeled Bn antagonist in the present study suggests that this, coupled with its high affinity, may make it a particularly useful ligand for such assays in the future.

In recent studies, antagonists for a number of types of

receptors, such as those for histamine (73), CCK (74), gastrin (74), adrenergic agents (75, 76), and cholinergic agents (35), have been shown to more clearly delineate receptor subtypes than do agonists. Binding studies based primarily on differential affinities for various agonists suggested that in the gastrointestinal tract as well as in the CNS at least two subtypes of Bn receptors exist (20–23), and this has been confirmed by recent cloning studies (24–26). One subtype is a GRP-preferring Bn receptor that has a high affinity for GRP or GRP-18–27 and a lower affinity for NMB (20, 24, 25). In *in situ* hybridization studies the GRP receptor was found to be widely distributed (25), and in binding studies it has been reported to be on isolated gastric smooth muscle cells (77), on pancreatic acinar cells (7, 16), in various areas of the CNS (18, 19), on 3T3 cells (10), and on small cell lung cancer cells (12). In contrast, the other subtype, the NMB receptor, has a high affinity for NMB and a lower affinity for GRP and GRP-18–27, has been identified as occurring widely in the CNS, and occurs on rat esophageal muscularis mucosa and isolated gastric smooth muscle cells (19, 20, 26, 77). Recent studies (22–25) present evidence that different classes of Bn receptor antagonists have differing selectivities for the two Bn receptor subtypes. In the present study the radiolabeled antagonist $^{125}$I-[D-Tyr$^{6}$]Bn(6–13)ME had a >10,000-fold higher affinity for the GRP receptors than for the NMB receptors, whereas the radiolabeled agonist had approximately equal affinities for both subtypes of Bn receptors. The availability of this radiolabeled antagonist should prove extremely useful in distinguishing GRP receptors from NMB receptors. At present, no high affinity radiolabeled antagonist with high selectivity for NMB receptors has been described, and at present this receptor can be distinguished in binding studies only by using radiolabeled agonists such as radiolabeled NMB (22).

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