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# Role of Receptor and Protein Kinase C Activation in the Internalization of the Gastrin-Releasing Peptide Receptor

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## ABSTRACT

The mechanisms regulating receptor internalization are not well understood and vary among different G protein-coupled receptors. The bombesin (Bn)/gastrin-releasing peptide receptor GRP-R, which is coupled to phospholipase C via the $G_q$ family of transducing proteins, is internalized rapidly after Bn binding. Agonist stimulation leads to rapid receptor phosphorylation, as does activation of protein kinase C (PKC) by phorbol-12-myristate-13-acetate (PMA). However, agonist- and PMA-induced phosphorylation occur at different receptor sites. Here, we examined the role of PKC in GRP-R internalization after agonist and antagonist binding. We synthesized [D-Tyr$^6$]Bn(6–13)propylamide ([D-Tyr$^6$]Bn(6–13)PA) and found that it potently inhibited Bn-stimulated insulin release and [$^{125}$I-Tyr$^4$]Bn binding ($K_i$ = 4.72 nM) in the HIT-T15 pancreatic cell line. The radiolabeled antagonist peptide, [$^{125}$I-D-Tyr$^6$]Bn(6–13)PA, bound with high affinity ($K_D$ = 0.29 nM at 4°) to a single class of receptor sites, and competition binding studies exhibited the analog specificity expected for the GRP-R subtype. Although the agonist [$^{125}$I-Tyr$^4$]Bn was internalized rapidly at 37° and subsequently degraded, [$^{125}$I-D-Tyr$^6$]Bn(6–13)PA was not internalized and was released into the medium mainly as intact peptide. The lysosomal inhibitor chloroquine (200 $\mu$M) increased the intracellular accumulation of [$^{125}$I-Tyr$^4$]Bn but had no effect on the subcellular distribution of [$^{125}$I-D-Tyr$^6$]Bn(6–13)PA. Consistent with these observations, the treatment of cells with 100 nM Bn at 37° reduced cell surface receptors within minutes, whereas [D-Tyr$^6$]Bn(6–13)PA had no effect. The addition of PMA did not induce the internalization of antagonist-occupied receptors, but pharmacological inhibition of PKC decreased the rate of agonist-induced receptor internalization. These results therefore demonstrate that although PKC contributes to agonist-induced internalization of the GRP-R, it does not elicit receptor internalization of the antagonist-occupied receptor.

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Ligand-induced receptor endocytosis is one of the mechanisms by which cells regulate receptor function; depleting the cell surface of receptors results in desensitization of the cellular response, whereas receptor recycling may play a role in receptor resensitization (Bohm *et al.*, 1997; Koenig and Edwardson, 1997). Despite its importance, the molecular signals required to initiate receptor internalization are not understood. For example, it is not clear how the nature of the bound ligand affects receptor endocytosis. Studies with select GPCRs indicated that agonist-occupied receptors are rapidly internalized while antagonist-occupied receptors remain on the cell surface after ligand binding (Bohm *et al.*, 1997; Koenig and Edwardson, 1997). In fact, agonist strength, as measured by the coupling efficiency of the agonist/receptor complex, correlates with the rate of agonist-induced sequestration of the $\beta$-adrenergic receptor (January *et al.*, 1997). However, studies with peptide-binding GPCRs have provided examples of antagonist-induced receptor internalization (Conchon *et al.*, 1994; Roettger *et al.*, 1997), as well as agonist-induced receptor endocytosis that is unrelated to agonist potency (Keith *et al.*, 1997, 1998). Further complexity is introduced by the fact that second-messenger-activated kinases can modulate receptor internalization (Liles *et al.*, 1986; Hoover and Toews, 1990; Fonseca *et al.*, 1995). In this study, we examined the role of the bound ligand and PKC activation in Bn receptor endocytosis.

The Bn family of structurally homologous peptides includes the two mammalian peptides GRP and neuromedin B and numerous amphibian homologs (Lebacq-Verheyden *et al.*, 1990; Nagalla *et al.*, 1996). In mammals, Bn-like peptides

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**ABBREVIATIONS:** GPCR, G protein-coupled receptor; Bn, bombesin; PA, propylamide; GRP, gastrin-releasing peptide; GRP-R, gastrin-releasing peptide receptor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HBSS, HEPES-buffered salt solution, pH 7.2; TFA, trifluoroacetic acid; PMA, phorbol-12-myristate-13-acetate; PKC, protein kinase C; GRK, G protein-coupled receptor kinase; PLC, phospholipase C; Gpp(NH)p, guanosine-5′-($\beta$,$\gamma$-imido)triphosphate.

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are found in both the central and peripheral nervous systems, as well as in endocrine cells in a number of tissues, including the gastrointestinal tract and the lung (Sunday *et al.*, 1988; Lebacq-Verheyden *et al.*, 1990). These peptides produce a diverse array of biological effects, including modulation of neuronal excitability and the regulation of smooth muscle contraction, cell proliferation, and exocrine and endocrine secretion, including pancreatic insulin release (Sunday *et al.*, 1988; Lebacq-Verheyden *et al.*, 1990). Bn-like peptides also promote cell proliferation and function as autocrine growth factors involved in the pathogenesis of small cell lung cancer (Sunday *et al.*, 1988; Lebacq-Verheyden *et al.*, 1990). Hence, Bn receptor antagonists are being vigorously investigated as inhibitors of tumor growth.

Three pharmacologically distinct Bn receptor subtypes have been cloned in mammals, all members of the GPCR family (Giladi *et al.*, 1993; Kroog *et al.*, 1995a). The GRP-preferring receptor, GRP-R, previously called BR1, binds both GRP and Bn with nanomolar affinity but binds neuromedin B only 1% as well. In contrast, the neuromedin B-preferring receptor binds neuromedin B with nanomolar affinity and GRP and Bn with 10- and 100-fold lower affinity, respectively. The BRS-3 receptor binds both GRP ($K_d \approx 300$ nM) and neuromedin B ($K_d \approx 20$ $\mu$M) with low affinity, and a naturally occurring, high affinity ligand has not been identified for this receptor subtype (Gorbulev *et al.*, 1992).

HIT-T15 is a clonal line of transformed pancreatic cells that retains many characteristics of normal $\beta$ cells (Santerre *et al.*, 1981), including increased insulin secretion on Bn stimulation (Swope and Schonbrunn, 1984, 1988). Bn initiates this response by binding to specific membrane receptors with high affinity for GRP (Swope and Schonbrunn, 1987), consistent with the known expression of the GRP-R in the pancreas (Battey *et al.*, 1991). In HIT-T15 cells, as in other responsive cell types, the GRP-R is coupled to PLC (Swope and Schonbrunn, 1988) via pertussis toxin-insensitive G proteins (Fischer and Schonbrunn, 1988). Activation of PLC causes a rapid increase in two second messengers, inositol trisphosphate and diacylglycerol (Swope and Schonbrunn, 1988; Regazzi *et al.*, 1990), which leads to a rise in cytosolic $Ca^{2+}$ and PKC activation (Swope and Schonbrunn, 1988; Regazzi *et al.*, 1990). These two signaling pathways act synergistically to induce a burst of insulin secretion in response to Bn (Swope and Schonbrunn, 1988). However, extended exposure to Bn results in desensitization and a loss in cell surface receptors, with the latter being at least partially responsible for the desensitization of HIT cells to further Bn stimulation (Swope and Schonbrunn, 1990). Bn stimulation of insulin secretion, as well as Bn-induced inositol trisphosphate production and intracellular $Ca^{2+}$ elevation, also are desensitized after a 2-hr exposure of cells to the PKC activator PMA (Swope and Schonbrunn, 1990). Although the complex molecular events involved in GRP-R desensitization are poorly understood, Bn binding and PKC activation by the phorbol ester PMA both stimulate the rapid phosphorylation of the GRP-R (Kroog *et al.*, 1995b; Williams *et al.*, 1996). Interestingly, Bn- and PMA-stimulated phosphorylation occurs at distinct sites (Williams *et al.*, 1996). Moreover, results with truncated and chimeric GRP-Rs have suggested that activations of both PKC- and second-messenger-independent kinases are required for normal receptor endocytosis (Benya *et al.*, 1993, 1994). Nevertheless, the role in GRP-R endocytosis for agonist-induced conformational changes during receptor activation versus signal transduction involving PKC stimulation remains unclear.

To elucidate the mechanisms involved in Bn receptor internalization, we synthesized a potent Bn receptor antagonist that can be radiolabeled. In the current study, we compare receptor-mediated processing of this antagonist with that of agonist by the endogenously expressed GRP-R in the HIT-T15 cell line and examine the role of PKC in receptor internalization.

## Experimental Procedures

**Materials.** Bn, [Tyr$^4$]Bn, [Leu$^{13}\Psi$(CH$_2$NH)Leu$^{14}$]Bn, neuromedin B, thyrotropin-releasing hormone, vasoactive intestinal peptide, epidermal growth factor, and somatostatin were from Bachem (Torrance, CA). PMA was from Sigma Chemical (St. Louis, MO). (±)-1-(5-Isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride and 1-(5-isoquinolinylsulfonyl)-piperazine hydrochloride were from LC Laboratories (Woburn, MA). Na$^{125}$I (specificity activity, 13.7 mCi/$\mu$g) was from Amersham (Arlington Heights, IL). Acetonitrile was from Baxter Healthcare (McGaw Park, IL). TFA was from Pierce (Rockford, IL). Sep-Pak C$_{18}$ cartridges were from Waters Associates (Milford, MA). Multiwell plates and culture flasks were from Corning Glassworks (Corning, NY). The 35-mm culture dishes were from Becton Dickinson Labware (Lincoln, NJ). F12 media and horse serum were from Grand Island Biological (Grand Island, NY). Fetal bovine serum was from JRH Biosciences (Lenexa, KS).

**Synthesis of [D-Tyr$^6$]Bn(6–13) and [D-Tyr$^6$]Bn(6–13)PA.** Synthesis of the peptides were performed by Dr. William T. Moore (University of Texas Medical School Analytical Chemistry Center, Houston, TX) using t-BOC/benzyl solid-phase methodology with an Applied Biosystems (ABI, Foster City, CA) model 430A automated peptide synthesizer. Peptides were cleaved and deblocked using either hydrogen fluoride alone for [D-Tyr$^6$]Bn(6–13) or propylamine and hydrogen fluoride for [D-Tyr$^6$]Bn(6–13)PA. Peptide structure was verified by amino acid analysis and fast atom bombardment mass spectrometry. Peptide purity was determined by analytical reverse-phase high performance liquid chromatography and shown to be >95%.

**Cell culture.** The establishment and properties of the HIT cell line have been described previously (Swope and Schonbrunn, 1984). Cells were grown as monolayer cultures in F12 media supplemented with 15% horse serum and 2.5% fetal bovine serum. For insulin release and peptide-binding experiments, the cells were seeded at a density of $3 \times 10^5$ cells/35-mm dish. The culture medium was changed every 3–4 days, and experiments were performed 1 day after a medium change. The cells were maintained for three to five medium changes before use.

**Measurement of insulin secretion.** Insulin release was determined as described previously (Swope and Schonbrunn, 1988, 1990). Briefly, cells were washed twice with HBSS, pH 7.2 (118 mM NaCl, 4.6 mM KCl, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 5 mM HEPES, 1 mg/ml bovine serum albumin, and 1 mg/ml $NaHCO_3$), and then incubated in a $CO_2$ incubator at 37° for 30 min. The buffer was replaced with 37° HBSS containing the appropriate concentration of peptide, and the cells were incubated for an additional 60 min. The buffer then was collected, centrifuged at $500 \times g$ for 10 min to remove any floating cells, and stored frozen. Radioimmunoassays were performed with guinea pig anti-insulin (porcine) serum as described previously (Swope and Schonbrunn, 1984).

**Measurement of peptide binding.** [D-Tyr$^6$]Bn(6–13)PA and [Tyr$^4$]Bn were individually radioiodinated using chloramine-T oxidation. Because the terminal methionine residue of [Tyr$^4$]Bn becomes oxidized during the iodination reaction, this was subsequently reduced according to the method of Vigna *et al.* (1988). Both radiolabeled peptides were purified by reverse-phase high performance

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liquid chromatography to a specific activity of 2200 Ci/mmol as described previously (Williams and Schonbrunn, 1994).

Binding studies were performed in ambient atmosphere as described previously (Swope and Schonbrunn, 1987). Briefly, cells were washed twice with HBSS without $NaHCO_3$ and then pre-equilibrated to the temperature of the binding reaction in 1 ml of fresh buffer. [$^{125}$I-D-Tyr$^6$]Bn(6–13)PA or [$^{125}$I-Tyr$^4$]Bn was added to each dish to a final concentration of $\sim 10^{-11}$ M. At the end of the appropriate incubation period, the buffer was aspirated and the dishes were rapidly rinsed with ice-cold 0.15 M NaCl. The cells then were dissolved in 0.1 N NaOH, and the cell-associated radioactivity was determined in a Pharmacia LKB (St. Quentin, France) $\gamma$ spectrometer at an efficiency of 75%. Specific binding was calculated as the difference between the amount of radioiodinated peptide bound in the absence (total binding) and the presence (nonspecific binding) of 100 nM Bn. Unless stated otherwise, the data shown represent specific binding and are given as the mean ± standard error.

An acid wash procedure was used to determine the cellular distribution of bound [$^{125}$I-D-Tyr$^6$]Bn(6–13)PA and [$^{125}$I-Tyr$^4$]Bn (Swope and Schonbrunn, 1987). After the binding incubation, cells were washed twice with ice-cold HBSS and treated for 5 min at 4° with 1 ml of 0.2 M acetic acid and 0.5 M NaCl, pH 2.5. Cells then were dissolved in 0.1 N NaOH, and the cell-associated radioactivity was determined.

To determine the effect of Bn and its analogs on receptor internalization, a milder acid wash procedure was used to dissociate unlabeled peptide before the binding reaction. Cells were preincubated with peptide at 37° for the indicated times to cause receptor internalization. The cells then were washed with ice-cold HBSS, treated for 5 min at 4° with 1 ml of HBSS containing 20 mM glycine, pH 3.0, and washed twice with ice-cold HBSS. The binding reaction subsequently was carried out with [$^{125}$I-Tyr$^4$]Bn at 4° for 4 hr as described. Specific [$^{125}$I-Tyr$^4$]Bn binding was 12,660 ± 1,470 cpm/dish (triplicate dishes) in control cells and 11,220 ± 1,100 cpm/dish in cells incubated with 100 nM Bn for 10 min at 4° and then washed with the pH 3.0 buffer before binding. In contrast, specific binding was 1,700 ± 170 cpm/dish in Bn-incubated cells washed with HBSS alone before binding. Therefore, HBSS containing 20 mM glycine, pH 3.0, is able to dissociate all prebound Bn without affecting the subsequent binding of radioligand to the receptor.

**Chromatography of $^{125}$I, $^{125}$I-tyrosine, and $^{125}$I-peptide.** To determine the nature of the radioactivity associated with the cells after the binding of radioiodinated peptide, cells were rinsed twice with ice-cold HBSS and immediately extracted in 1 ml of 0.1 N HCl and 0.1% bovine serum albumin for 10 min at 4° or incubated in fresh HBSS for 1 hr at 37°. The nature of the radiolabeled material extracted from the cells or dissociated into the buffer was analyzed as described previously (Swope and Schonbrunn, 1987). Briefly, samples were applied to Sep-Pak $C_{18}$ cartridges that had been washed with 5 ml of methanol followed by 10 ml of deionized water. Iodide ($^{125}I^-$), $^{125}$I-tyrosine, and $^{125}$I-peptides were sequentially eluted with 15 ml of 0.1% TFA, 15 ml of 25% methanol in 0.1% TFA, and 15 ml of 80% methanol in 0.1% TFA, respectively.

**Data analysis.** Data analysis was performed with the programs Multifit (Day Computing, Milton, Cambridge, UK) or D/R (D. L. Steffen, Biomedical Computing, Houston, TX) as described previously (Williams and Schonbrunn, 1994) and according to the method of Cheng and Prusoff (1973).

## Results

**Design and characterization of a Bn receptor antagonist.** Carboxyl-terminal des-methionine alkylamide and ester analogs of Bn are the most potent known antagonists for the GRP-R and exhibit >500-fold selectivity for the GRP-R relative to the neuromedin B-preferring receptor subtype (Lin *et al.*, 1995). Among the highest affinity compounds of this class is [D-Phe$^6$]Bn(6–13)PA (Table 1), which inhibits Bn-stimulated amylase release from guinea pig pancreatic acini, Swiss 3T3 cell proliferation, and [$^{125}$I-Tyr$^4$]Bn binding to the GRP-R with an $EC_{50}$ value in the nanomolar range (Wang *et al.*, 1990). For our studies, we needed a potent antagonist that could be radioiodinated; therefore, we synthesized a peptide in which D-Tyr was substituted for D-Phe in [D-Phe$^6$]Bn(6–13)PA. Table 1 compares the structure of this derivative to several natural and synthetic Bn analogs, including [Leu$^{13}\Psi$($CH_2$NH)Leu$^{14}$]Bn, a less potent GRP-R antagonist (Lin *et al.*, 1995).

Both [D-Tyr$^6$]Bn(6–13) and its propylamide derivative were tested for their ability to inhibit [$^{125}$I-Tyr$^4$]Bn binding to HIT cells (Fig. 1). The $K_d$ values obtained from several such competition experiments (Table 2) showed that the binding affinity for [D-Tyr$^6$]Bn(6–13)PA was seven times lower than that for Bn. However, [Leu$^{13}\Psi$($CH_2$NH)Leu$^{14}$]Bn, neuromedin B, and [D-Tyr$^6$]Bn(6–13) were all ≥200-fold less potent than Bn. The observed affinity and specificity for Bn compared with neuromedin B demonstrate that HIT cells contain the GRP-R subtype (Von Schrenck *et al.*, 1989; Jensen and Coy, 1991). Both of the tyrosine-substituted Bn(6–13) analogs bound to the HIT cell receptor, with the propylamide exhibiting the higher affinity.

To determine whether the [D-Tyr$^6$]Bn(6–13) derivatives functioned as antagonists, both the free acid and the propylamide were tested for inhibition of Bn-stimulated insulin secretion. As shown in Fig. 2, 3 nM Bn stimulated insulin secretion ~4.8-fold. In contrast, the [D-Tyr$^6$]Bn(6–13) peptides had no effect at a concentration of 10 $\mu$M. However, both [D-Tyr$^6$]Bn(6–13)PA and [D-Tyr$^6$]Bn(6–13) produced a dose-dependent inhibition of Bn-stimulation (Fig. 2). In two experiments, [D-Tyr$^6$]Bn(6–13)PA and [D-Tyr$^6$]Bn(6–13) decreased Bn-stimulated insulin secretion with $EC_{50}$ values of 128 ± 37 and 4797 ± 1026 nM (mean ± range), respectively. Because [D-Tyr$^6$]Bn(6–13)PA was the more potent antagonist, we radioiodinated and characterized this peptide further.

**Binding properties of the radiolabeled Bn antagonist.** Both the rate of [$^{125}$I-D-Tyr$^6$]Bn(6–13)PA binding and the amount of peptide bound to HIT cells at equilibrium

TABLE 1
Amino acid sequences of Bn analogs

| Peptide | Amino acid sequence |
|---|---|
| Bn | pGlu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-$NH_2$ |
| GRP(14–27) | Met-Tyr-Pro-Arg---------His----------$NH_2$ |
| [D-Phe$^6$]Bn(6–13)PA | DPhe----------$NHC_3H_7$ |
| [D-Tyr$^6$]Bn(6–13) | DTyr----------COOH |
| [D-Tyr$^6$]Bn(6–13)PA | DTyr----------$NHC_3H_7$ |
| Neuromedin B | --------Leu---------Thr---------Phe-----$NH_2$ |
| [Leu$^{13}\Psi$ ($CH_2$NH) Leu$^{14}$]Bn | ----------$\Psi$ Leu-$NH_2$ |

Amino acid identity with bombesin is indicated by a dashed line. PA denotes propylamide.

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varied with temperature (Fig. 3). At 4°, maximal binding was attained at 60 min and remained stable up to 180 min. At 37°, [$^{125}$I-D-Tyr$^{6}$]Bn(6–13)PA binding was maximal by 15 min and was maintained for 60 min.

The concentration dependence for [$^{125}$I-D-Tyr$^{6}$]Bn(6–13)PA binding to HIT cells is shown in Fig. 4. Binding was saturable and temperature dependent. In two replicate experiments, [$^{125}$I-D-Tyr$^{6}$]Bn(6–13)PA bound to a single class of noninteracting sites with $K_d$ values of 0.29 ± 0.01 and 0.48 ± 0.02 nM at 4° and 37°, respectively. The maximum binding capacity for antagonist was 5515 ± 1465 sites/cell at 4° and 7315 ± 2006 sites/cell at 37° (mean ± range, two determinations).

To determine the specificity of [$^{125}$I-D-Tyr$^{6}$]Bn(6–13)PA for the Bn receptor, a number of peptides were tested for their ability to compete for binding. The structurally unrelated peptides vasoactive intestinal peptide, thyrotropin-releasing hormone, somatostatin, and epidermal growth factor, at concentrations of 100 nM, did not inhibit [$^{125}$I-D-Tyr$^{6}$]Bn(6–13)PA binding by >4% (data not shown), whereas 100 nM Bn inhibited binding by 82% (Fig. 5). Moreover, five Bn analogs competed for [$^{125}$I-D-Tyr$^{6}$]Bn(6–13)PA binding with the same rank order of potency as for [$^{125}$I-Tyr$^{4}$]Bn binding (Fig. 5 and Table 2). Taken together, our results demonstrate that [$^{125}$I-D-Tyr$^{6}$]Bn(6–13)PA specifically binds to a GRP-R in HIT cells.

**Comparison of receptor-mediated processing of agonist and antagonist.** The results in Fig. 3 showed that [$^{125}$I-D-Tyr$^{6}$]Bn(6–13)PA binding is maintained at a steady state between 15 and 60 min at 37°. In contrast, [$^{125}$I-Tyr$^{4}$]Bn binding to HIT cells at 37° reaches a maximum at 45 min and then falls rapidly (Swope and Schonbrunn, 1987). The decrease in [$^{125}$I-Tyr$^{4}$]Bn binding is due to the internalization and degradation of bound [$^{125}$I-Tyr$^{4}$]Bn concomitant with receptor sequestration (Swope and Schonbrunn, 1987). Therefore, we investigated whether the difference in the binding time course for [$^{125}$I-D-Tyr$^{6}$]Bn(6–13)PA and [$^{125}$I-Tyr$^{4}$]Bn resulted from a difference in the receptor-mediated processing of the two ligands (Fig. 6).

After equilibrium binding at 4°, most of the cell-associated [$^{125}$I-D-Tyr$^{6}$]Bn(6–13)PA (Fig. 6, *top*) and [$^{125}$I-Tyr$^{4}$]Bn (Fig. 6, *bottom*) were removed by an acid wash. When the temperature was raised to 37°, the amount of receptor-bound antagonist that was resistant to acid did not change during a 20-min incubation (Fig. 6, *top*). However, the total amount of cell-associated [$^{125}$I-D-Tyr$^{6}$]Bn(6–13)PA steadily decreased during this time and accumulated in the medium. By comparison, 55% of the receptor-bound [$^{125}$I-Tyr$^{4}$]Bn became resistant to acid dissociation within 3 min at 37° (Fig. 6, *bottom*). During this time, the total amount of cell-associated [$^{125}$I-Tyr$^{4}$]Bn did not change: the amount of radiolabel in the medium began to increase only after 5 min. Together, these data show that receptor-bound [$^{125}$I-D-Tyr$^{6}$]Bn(6–13)PA is not internalized at 37°, whereas [$^{125}$I-Tyr$^{4}$]Bn is internalized rapidly. The 3-min lag before radioactivity appeared in the medium after [$^{125}$I-Tyr$^{4}$]Bn binding is presumably due to the time required for agonist endocytosis and processing before release from the cell (Swope and Schonbrunn, 1987). Consistent with this interpretation, a lag was not observed with [$^{125}$I-D-Tyr$^{6}$]Bn(6–13)PA dissociation.

Previous studies in HIT cells demonstrated that [$^{125}$I-Tyr$^{4}$]Bn is degraded in lysosomes and deiodinated, and the radiolabel is subsequently released from the cell as free [$^{125}$I]iodide (Swope and Schonbrunn, 1987). Therefore, we next determined how receptor-bound [$^{125}$I-D-Tyr$^{6}$]Bn(6–13)PA was metabolized. After incubating cells with [$^{125}$I-D-Tyr$^{6}$]Bn(6–13)PA or [$^{125}$I-Tyr$^{4}$]Bn at 4°, 92% of the specifically bound radioactivity released from the cell surface by an acid wash chromatographed as intact peptide on Sep-Pak $C_{18}$ columns (Table 3). When the temperature was raised to 37° for 60 min, 60% of the radioactivity released into the buffer in the [$^{125}$I-D-Tyr$^{6}$]Bn(6–13)PA group chromatographed as intact peptide and 33% chromatographed as [$^{125}$I]tyrosine (Table 3). In contrast, essentially all of the radioactivity released from the [$^{125}$I-Tyr$^{4}$]Bn group was degraded, mostly to iodide (Table 3). These results demonstrate that [$^{125}$I-D-Tyr$^{6}$]Bn(6–13)PA is degraded more slowly than [$^{125}$I-Tyr$^{4}$]Bn. Moreover,

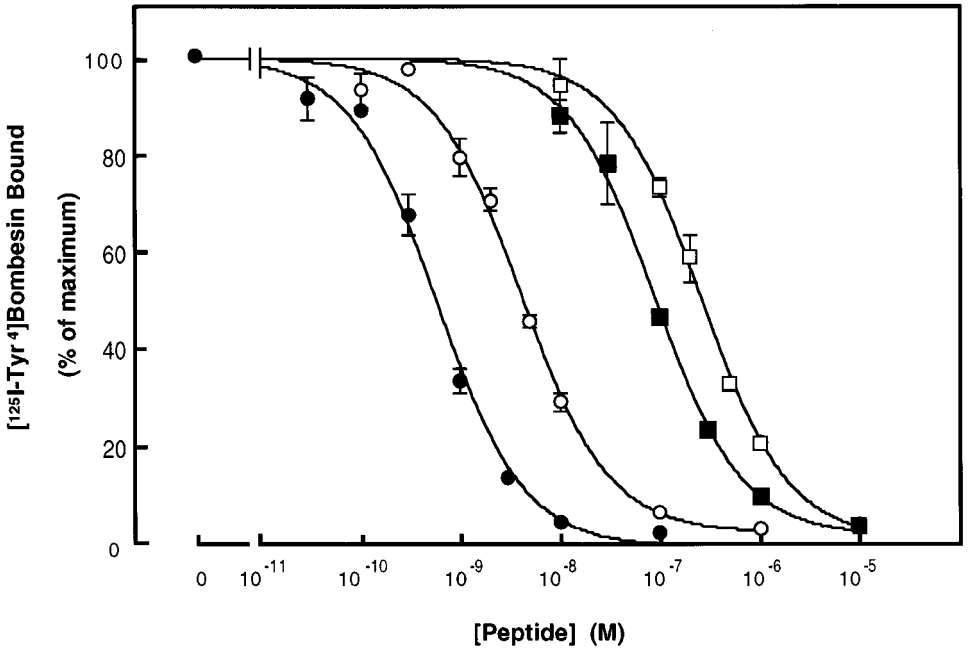


**Fig. 1.** Competition for [$^{125}$I-Tyr$^{4}$]Bn binding by Bn analogs. HIT cells (4 × $10^{6}$/dish) were incubated at 4° for 15 hr with [$^{125}$I-Tyr$^{4}$]Bn (7 × $10^{4}$ cpm/ml, $10^{-11}$ M) and various concentrations of unlabeled Bn (●), [D-Tyr$^{6}$]Bn(6–13)PA (○), [Leu$^{13}$Ψ(CH$_2$NH)Leu$^{14}$]Bn (■), or [D-Tyr$^{6}$]Bn(6–13) (□). The total cell-associated radioactivity at each concentration of unlabeled peptide was calculated as a percentage of [$^{125}$I–Tyr$^{4}$]Bn bound in the absence of any competitor (1.8 × $10^{4}$ cpm/dish). *Points*, mean of triplicate dishes. *Bars*, mean ± standard error. *Curves*, computer-fitted regression lines with the following values for the $EC_{50}$: Bn, 0.59 ± 0.08 nM; [D-Tyr$^{6}$]Bn(6–13)PA, 4.16 ± 0.43 nM; [Leu$^{13}$Ψ(CH$_2$NH)Leu$^{14}$]Bn, 89 ± 16 nM; and [D-Tyr$^{6}$]Bn(6–13), 261 ± 34 nM.

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the different degradation products formed from the two peptides suggests that distinct mechanisms are involved.

To further investigate receptor-mediated ligand processing, we used chloroquine to inhibit lysosomal proteinases (Lie and Schofield, 1973). HIT cells were incubated at 4° with either [$^{125}$I-D-Tyr$^6$]Bn(6–13)PA or [$^{125}$I-Tyr$^4$]Bn to allow cell surface binding, and the temperature then was raised to 37° for 30 min (Fig. 7). Both the binding and dissociation incubations were performed in the presence or absence of chloroquine. There was no difference in the intracellular distribution of radioactivity between the control and chloroquine-treated groups with [$^{125}$I-D-Tyr$^6$]Bn(6–13)PA (Fig. 7A). After 30 min at 37°, 76% of the initially bound radioactivity was released into the buffer in both groups, 20% was acid sensitive (cell surface associated), and 4% was acid resistant. In contrast, chloroquine inhibited the release of radioactivity from bound [$^{125}$I-Tyr$^4$]Bn by 50% and increased intracellular [$^{125}$I-Tyr$^4$]Bn 3-fold (Fig. 7B). These results demonstrate that unlike [$^{125}$I-Tyr$^4$]Bn, the processing of [$^{125}$I-D-Tyr$^6$]Bn(6–13)PA is not chloroquine sensitive, indicating that [$^{125}$I-D-Tyr$^6$]Bn(6–13)PA is not routed through lysosomes.

TABLE 2

Potencies of Bn analogs to compete for [$^{125}$I-Tyr$^4$]Bn or [$^{125}$I-D-Tyr$^6$]Bn (6–13)PA binding

The equilibrium dissociation constant ($K_d$) of each Bn analogue was calculated according to Cheng and Prusoff (1973) from the $EC_{50}$ of [$^{125}$I-Tyr$^4$] Bn and [$^{125}$I-D-Tyr$^6$]/bombesin (6–13) PA binding obtained from experiments such as those shown in Figs. 1 and 5, respectively. The values represent the mean ± standard error and the number of replicate experiments for each analog are shown in parentheses.

| Competing peptide | $K_d$ | |
|---|---|---|
| | [$^{125}$I-Tyr$^4$]Bn | [$^{125}$I-D-Tyr$^6$]Bn (6–13) PA |
| | *nM* | |
| Agonist | | |
| Bn | 0.50 ± 0.10 (3) | 0.55 ± 0.02 (3) |
| Neuromedin B | 183 ± 4 (2) | 74 ± 2 (2) |
| Antagonist | | |
| [Leu$^{13}$Ψ(CH$_2$NH)Leu$^{14}$]Bn | 118 ± 18 (3) | 38 ± 8 (2) |
| [D-Tyr$^6$]Bn(6–13) | 331 ± 88 (3) | 112 ± 20 (3) |
| [D-Tyr$^6$]Bn(6–13)PA | 4.7 ± 0.8 (3) | 2.0 ± 0.2 (3) |

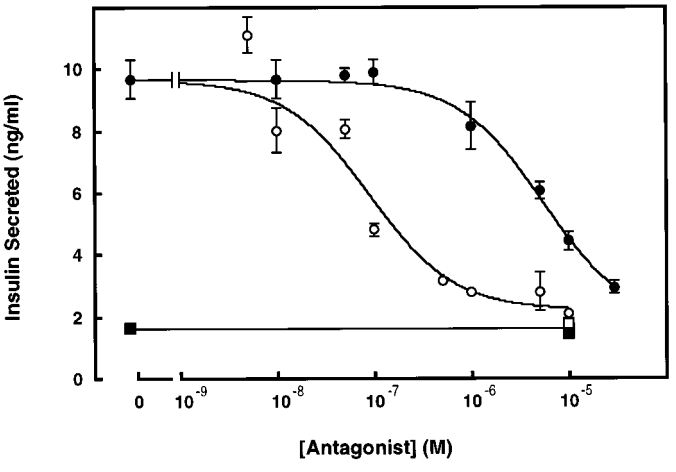


**Fig. 2.** Effect of Bn analogs on insulin secretion. HIT cells (4 × 10$^6$/dish) were incubated at 37° for 60 min with the indicated concentrations of [D-Tyr$^6$]Bn(6–13)PA (□), [D-Tyr$^6$]Bn(6–13) (■), 3 nM Bn plus [D-Tyr$^6$]Bn(6–13)PA (○), or 3 nM Bn plus [D-Tyr$^6$]Bn(6–13) (●). The insulin accumulated in the buffer was subsequently measured by radioimmunoassay. *Points*, the mean insulin secreted in triplicate dishes. *Bars*, mean ± standard error. *Curves*, computer-fitted regression lines with an $EC_{50}$ value of 91 ± 27 and 5520 ± 1160 nM for [D-Tyr$^6$]Bn(6–13)PA and [D-Tyr$^6$]Bn(6–13), respectively.

**Comparison of agonist and antagonist receptor regulation.** To compare the effect of antagonist and agonist on receptor internalization, HIT cells were incubated with 100 nM concentration of either Bn or [D-Tyr$^6$]Bn(6–13)PA at 37°. At the times indicated (Fig. 8), the cells were washed with HBSS containing 20 mM glycine, pH 3, which dissociates >95% of the surface-bound peptide without affecting the rebinding of ligand to the receptor (see Experimental Procedures). After this acid wash, binding of [$^{125}$I-Tyr$^4$]Bn to cell surface receptors was measured at 4°. The results in Fig. 8 show that pretreatment with 100 nM [D-Tyr$^6$]Bn(6–13)PA at 37° for up to 60 min did not decrease receptor binding. In contrast, incubation with 100 nM Bn caused a 56% reduction in binding by 5 min, and this decrease was maintained for 60 min (Fig. 8). Therefore, unlike the agonist, receptor occupancy with the antagonist [D-Tyr$^6$]Bn(6–13)PA did not decrease cell surface receptors, demonstrating that receptor occupancy alone is not sufficient to induce receptor internalization.

Binding of Bn leads to the generation of diacylglycerol and consequently to the activation of PKC (Swope and Schonbrunn, 1988). However, direct activation of PKC by phorbol ester treatment does not cause the internalization of unoccupied Bn receptors in HIT cells (Swope and Schonbrunn, 1990). To determine whether activation of PKC stimulates the internalization of antagonist-occupied receptors, HIT cells were preincubated for 15 min at 37° with 100 nM PMA and then treated with 100 nM [D-Tyr$^6$]Bn(6–13)PA in the continued presence of PMA. Treatment with the combination of PMA and [D-Tyr$^6$]Bn(6–13)PA for up to 60 min did not affect cell surface receptor binding (Fig. 8). Thus, PKC activation does not promote internalization of the antagonist-occupied receptor.

To determine whether PKC activation is necessary for receptor internalization of agonist-occupied receptors, cells were preincubated for 15 min at 37° with the PKC inhibitor 4 μM GF109203X (Toullec *et al.*, 1991). Bn (100 nM) then was added to induce receptor internalization. After 10 min, the cells were washed with pH 3 buffer and incubated with [$^{125}$I-D-Tyr$^6$]Bn(6–13)PA at 4° to quantify the cell surface

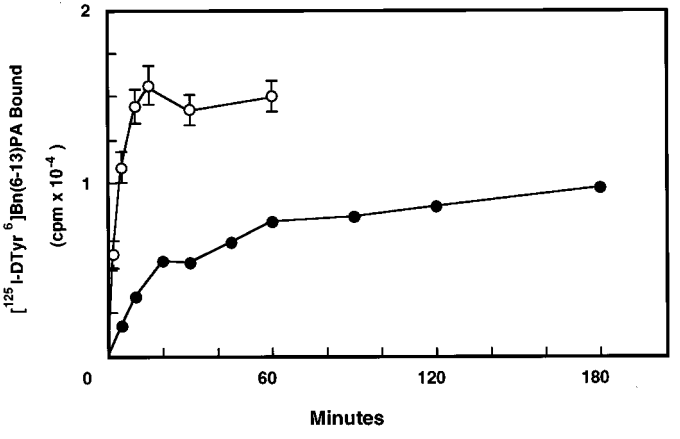


**Fig. 3.** Time course of [$^{125}$I-D-Tyr$^6$]Bn(6–13)PA binding. HIT cells (4 × 10$^6$/dish) were incubated either at 4° (●) or at 37° (○) with [$^{125}$I-D-Tyr$^6$]Bn(6–13)PA (7 × 10$^4$ cpm/ml, 10$^{-11}$ M). At the times shown, specific binding was determined as described in Experimental Procedures.

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receptors remaining. Treatment of cells with 100 nM Bn for 10 min at 37° decreased [$^{125}$I-D-Tyr$^{6}$]Bn(6–13)PA binding to the same extent in the presence and absence of GF109203X (data not shown). Because at 37° GRP-R internalization occurred very rapidly, we further examined the effect of GF109203X on receptor internalization at 30°. As shown in Fig. 9, GF109203X decreased the rate of Bn-induced GRP-R internalization. However, after a 10-min incubation with Bn at 30°, there was no difference in cell surface receptors between the control and inhibitor groups (data not shown). These results demonstrate that activation of PKC increases the rate of agonist-induced GRP-R internalization but is not essential for induction of endocytosis.

## Discussion

Both second-messenger formation and stimulation of biological responses, such as insulin secretion, desensitize rapidly in the continued presence of Bn (Swope and Schonbrunn, 1987, 1990; Kroog *et al.*, 1995a). Agonist-induced internalization of plasma membrane receptors has been proposed to play a role in this desensitization process in HIT cells because the

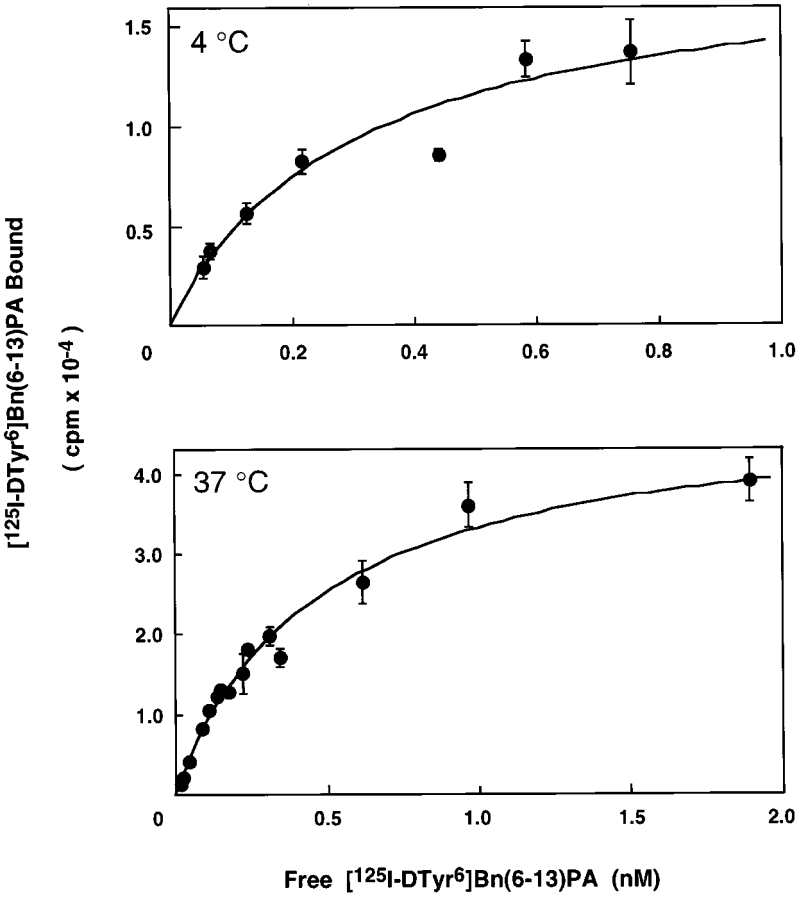


**Fig. 4.** Concentration dependence of [$^{125}$I-D-Tyr$^{6}$]Bn(6–13)PA binding. HIT cells (1 × 10$^{6}$/dish) were incubated with the indicated concentrations of [$^{125}$I-D-Tyr$^{6}$]Bn(6–13)PA either at 4° for 90 min (*top*) or at 37° for 30 min (*bottom*). *Points*, mean ± standard error of the specific binding observed in triplicate dishes. *Curves*, computer-fitted regression lines. The fitted value for the $K_d$ is 0.29 ± 0.01 nM at 4° and 0.48 ± 0.02 nM at 37°. The maximal binding capacity is 5500 ± 1500 sites/cell at 4° and 7300 ± 2000 sites/cell at 37°.

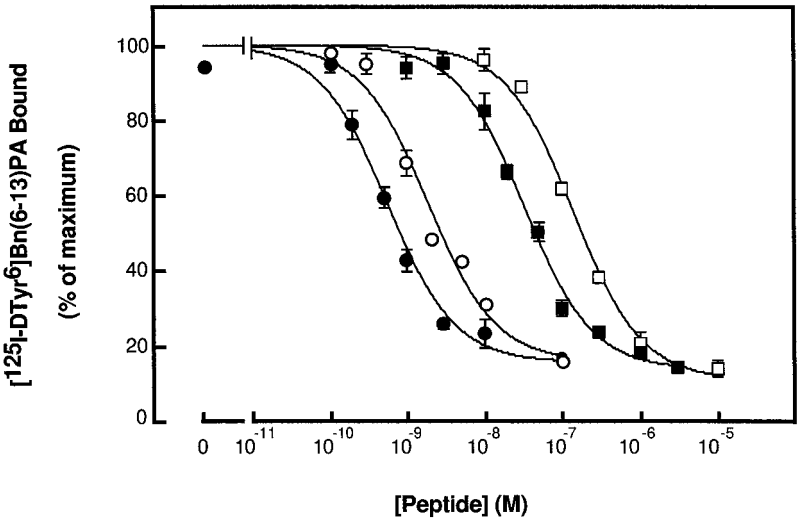


**Fig. 5.** Competition for [$^{125}$I-D-Tyr$^{6}$]Bn(6–13)PA binding by Bn analogs. HIT cells (4 × 10$^{6}$/dish) were incubated at 4° for 2 hr with [$^{125}$I-D-Tyr$^{6}$]Bn(6–13)PA (7 × 10$^{4}$ cpm/ml) and various concentrations of unlabeled Bn (●), [D-Tyr$^{6}$]Bn(6–13)PA (○), [Leu$^{13}$Ψ(CH$_2$NH)Leu$^{14}$]Bn (■), or [D-Tyr$^{6}$]Bn(6–13) (□). The total cell-associated radioactivity at each concentration of unlabeled peptide was calculated as a percentage of [$^{125}$I-D-Tyr$^{6}$]Bn(6–13)PA bound in the absence of any competitor (2 × 10$^{3}$cpm/dish). *Points*, represents the mean of triplicate dishes. *Bars*, mean ± standard error. *Curves*, computer-fitted regression lines with EC$_{50}$ values of 0.56 ± 0.10 nM for Bn, 1.92 ± 0.29 nM for [D-Tyr$^{6}$]Bn(6–13)PA, 33 ± 4 nM for [Leu$^{13}$Ψ(CH$_2$NH)Leu$^{14}$]Bn, and 141 ± 23 nM for [D-Tyr$^{6}$]Bn(6–13).

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rate of Bn-induced receptor sequestration is identical to the rate of desensitization, both occurring within minutes of agonist binding (Swope and Schonbrunn, 1990). Although the molecular mechanisms involved in GRP-R regulation are not known, the time course for Bn-induced receptor phosphorylation correlates with receptor internalization and desensitization (Kroog *et al.*, 1995b; Williams *et al.*, 1996). Phosphorylation of the GRP-R after agonist binding is a complex process mediated by two classes of protein kinases: a 7-hydroxy-staurosporine-sensitive PKC that is activated by the diacylglycerol produced on Bn stimulation and a 7-hydroxy-staurosporine-insensitive kinase, presumably a member of the GRK family (Kroog *et al.*, 1995b; Williams *et al.*, 1996). GRKs are serine and threonine kinases that preferentially phosphorylate the agonist-occupied, activated conformation of seven-transmembrane-domain receptors and thereby cause receptor/G protein uncoupling and receptor endocytosis (Haga *et al.*, 1994; Premont *et al.*, 1995). Phosphorylation of the GRP-R by the PKC and non-PKC mechanisms occurs at distinct sites because a receptor antibody that is unable to recognize the receptor after PMA-stimulated phosphorylation immunoprecipitates $^{32}PO_4$-labeled receptor after Bn treatment (Williams *et al.*, 1996). Antagonists do not stimulate GRP-R phosphorylation (Kroog *et al.*, 1995b), presumably because they neither induce the activated receptor conformation necessary for phosphorylation by GRKs nor lead to second-messenger formation. Hence, they provide useful tools to dissect the mechanisms involved in ligand-stimulated receptor internalization and sequestration.

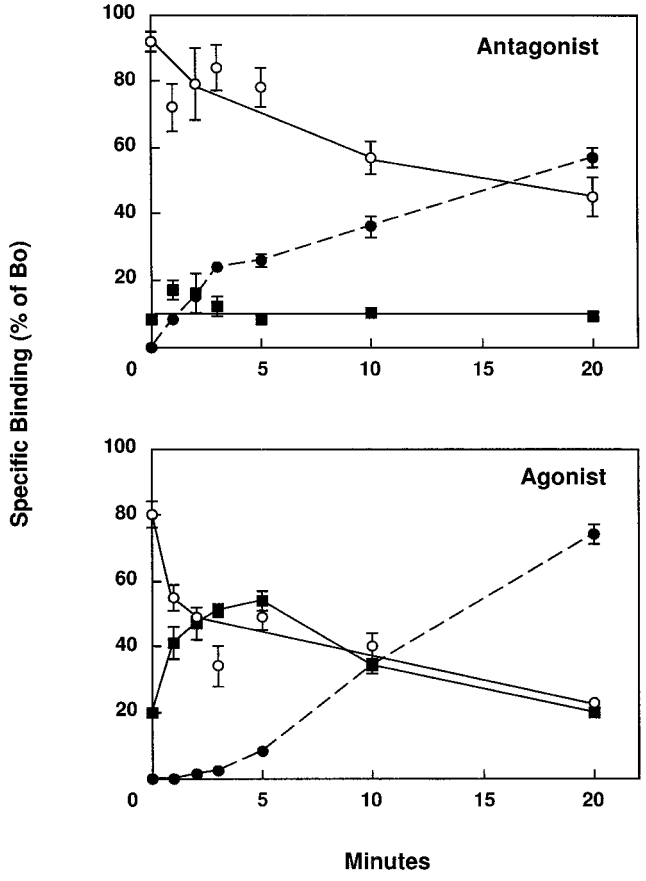


**Fig. 6.** Cellular distribution of receptor bound [$^{125}$I-D-Tyr$^6$]Bn(6–13)PA and [$^{125}$I-Tyr$^4$]Bn. HIT cells (4 × 10$^6$/dish) were incubated at 4° with either [$^{125}$I-D-Tyr$^6$]Bn(6–13)PA (7 × 10$^4$ cpm/ml) for 1 hr (*top*) or with [$^{125}$I-Tyr$^4$]Bn (7 × 10$^4$ cpm/ml) for 2 hr (*bottom*) in both the absence or presence of 100 nM unlabeled Bn. At t = 0, the cells were rapidly washed with cold saline and then incubated at 37° in 1 ml of HBSS to allow receptor-mediated processing of ligand to occur. At the times indicated, the buffer was collected, and the cells were treated immediately with pH 2.5 buffer for 5 min and then dissolved in NaOH. The radioactivity released into the buffer (●), the acid-sensitive radioactivity (○), and the radioactivity remaining with the cells (■) were measured. Values are shown as a percentage of the specific binding at t = 0 ($B_o$), which was 5.7 × 10$^3$ cpm/dish with [$^{125}$I-D-Tyr$^6$]Bn(6–13)PA and 1.8 × 10$^4$ cpm/dish with [$^{125}$I-Tyr$^4$]Bn.

In the current report, we show that [D-Tyr$^6$]Bn(6–13)PA is a specific antagonist that recognizes the GRP-R with high affinity. [D-Tyr$^6$]Bn(6–13)PA by itself did not affect insulin secretion in HIT cells; rather, it caused a dose-dependent inhibition of Bn-stimulated secretion. Its affinity for the GRP-R ($K_d \approx 3$ nM) was similar to that reported for the Phe$^6$ analog [D-Phe$^6$]Bn(6–13)PA ($K_d$ = 4.4 nM) (Wang *et al.*, 1990). Binding of the radiolabeled antagonist [$^{125}$I-D-Tyr$^6$]Bn(6–13)PA was rapid, time and temperature dependent, reversible, and saturable. Only Bn and peptides that interact with Bn receptors inhibited [$^{125}$I-D-Tyr$^6$]Bn(6–13)PA binding, whereas agents that interact with other peptide receptors, such as vasoactive intestinal peptide, somatostatin, thyrotropin-releasing hormone and epidermal growth factor, had no effect. Furthermore, the affinities of the various Bn receptor agonists and antagonists calculated from competition binding studies with the antagonist [$^{125}$I-D-Tyr$^6$]Bn(6–13)PA agreed closely with those obtained with the agonist [$^{125}$I-Tyr$^4$]Bn. Thus, the two radiolabeled peptides identify the same receptor. The relative affinity of this receptor for Bn compared with neuromedin B showed that the radioligands were binding to a GRP-R subtype. Receptor affinity for [$^{125}$I--Tyr$^6$]Bn(6–13)PA at 4° ($K_d$ = 0.3 nM) was 10 times higher than that for the uniodinated peptide ($K_d$ = 3 nM). Thus [$^{125}$I-D-Tyr$^6$]Bn(6–13)PA provides a new high af-

TABLE 3

Degradation of [$^{125}$I-D-Tyr$^6$]Bn(6–13)PA and [$^{125}$I-Tyr$^4$]Bn

HIT cells (4 × 10$^6$/dish) were incubated at 4° with [$^{125}$I-D-Tyr$^6$] Bn (6–13) PA for 1 hr or with [$^{125}$I-Tyr$^4$] Bn for 4 hr. The cells were rapidly washed with saline (t = 0 min) and either extracted at 4° for 10 min with 0.1 M HCl/0.1% BSA (cell extract) or incubated at 37° for 60 min in fresh buffer. Both the cell extract and buffer samples were analyzed by chromatography on Sep-Pak $C_{18}$ cartridges as described in the text. The radioactivity in each fraction was calculated as a percentage of the total radioactivity present in the sample.

| | Sample | Time | Fraction | | |
|---|---|---|---|---|---|
| | | | $^{125}$I | [$^{125}$I]Tyrosine | [$^{125}$I]Peptide |
| | | *min* | | *% of Total $^{125}$I* | |
| [$^{125}$I-D-Tyr$^6$]Bn(6–13)PA | Cell extract | 0 | 4 ± 3 | 3 ± 1 | 92 ± 6 |
| | Buffer | 60 | 7 ± 0.3 | 33 ± 1 | 60 ± 0.5 |
| [$^{125}$I-Tyr$^4$]Bn | Cell extract | 0 | 5 ± 1 | 3 ± 1 | 92 ± 2 |
| | Buffer | 60 | 91 ± 1 | 6 ± 1 | 3 ± 0.7 |

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finity ligand for the GRP-R; iodination actually increases its affinity for the receptor.

We used two different assays to compare agonist- and antagonist-induced receptor internalization. First, we measured changes in the distribution of radiolabeled, receptor-bound ligand using low pH to remove cell surface-associated peptide. Second, we assessed receptor sequestration by measuring cell surface binding at 4° after pretreatment with unlabeled ligand. In the first experimental situation, receptor endocytosis is being measured at low receptor occupancy under conditions where second-messenger formation is increased only slightly. In the latter paradigm, receptors are fully occupied by peptide and second-messenger production is maximally stimulated.

Unlike [$^{125}$I-Tyr$^{4}$]Bn, the radiolabeled antagonist [$^{125}$I-D-Tyr$^{6}$]Bn(6–13)PA was not internalized after receptor binding in HIT cells. This observation confirms previous results showing that the related antagonist [$^{125}$I-D-Tyr$^{6}$]Bn(6–13)ME was minimally internalized by GRP-Rs in other cell types (Mantey *et al.*, 1993; Tseng *et al.*, 1995). We further showed that receptor-bound [$^{125}$I-Tyr$^{4}$]Bn and [$^{125}$I-D-Tyr$^{6}$]Bn(6–13)PA were degraded to a different extent and to different products. The agonist was completely hydrolyzed to iodide before release from cells, whereas the antagonist was released mainly as intact peptide with only partial degradation to iodotyrosine. Moreover, chloroquine inhibited the release of [$^{125}$I-Tyr$^{4}$]Bn degradation products and increased the intracellular accumulation of this peptide, whereas it did not alter the cellular distribution or the release of receptor-bound [$^{125}$I-D-Tyr$^{6}$]Bn(6–13)PA. Thus, unlike agonist, [$^{125}$I-D-Tyr$^{6}$]Bn(6–13)PA is not routed through lysosomes via a receptor-mediated internalization process. Any degradation of the bound antagonist peptide must occur at the cell surface, perhaps catalyzed by plasma membrane endopeptidases (Bunnett *et al.*, 1985). Consistent with these results, pretreatment of HIT cells with saturating concentrations of the unlabeled antagonist did not produce receptor sequestration. As described previously (Swope and Schonbrunn, 1990), Bn

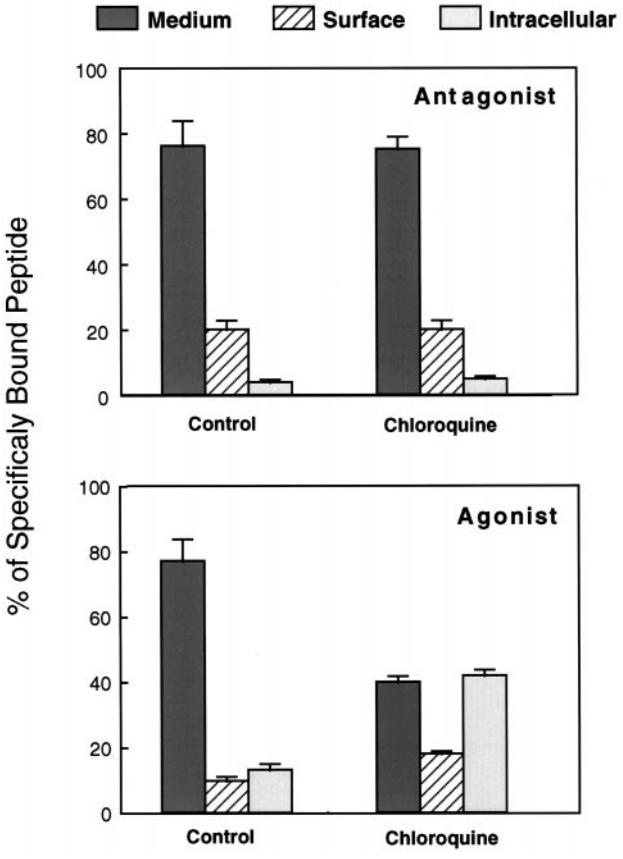


**Fig. 7.** Effect of chloroquine on receptor-mediated processing of [$^{125}$I-D-Tyr$^{6}$]Bn(6–13)PA and [$^{125}$I-Tyr$^{4}$]Bn. HIT-T15 cells ($4 \times 10^{6}$/dish) were preincubated at 37° for 20 min in HBSS with or without 200 $\mu$M chloroquine. The cells were chilled and incubated at 4° in the continued presence or absence of chloroquine with [$^{125}$I-D-Tyr$^{6}$]Bn(6–13)PA ($7 \times 10^{4}$ cpm/ml, $10^{-11}$ M) for 1 hr (*top*) or with [$^{125}$I-Tyr$^{4}$]Bn ($7 \times 10^{4}$ cpm/ml, $10^{-11}$ M) for 2 hr (*bottom*). After the binding incubation, the cells were rapidly washed and incubated at 37° in 1 ml of fresh buffer with or without chloroquine. After 30 min, the buffer was collected (*shaded*) and the cells were treated with cold 0.2 M acetic acid/0.5 M NaCl, pH 2.5, for 5 min. The radioactivity in both the acid wash (*striped*) and in a NaOH extract of the cells (*open*) was measured and expressed as a percentage of the specifically bound trace recovered in all the fractions ($4.1 \times 10^{3}$ cpm/dish for [$^{125}$I-D-Tyr$^{6}$]Bn(6–13)PA and $8.3 \times 10^{3}$ cpm/dish for [$^{125}$I-Tyr$^{4}$]Bn).

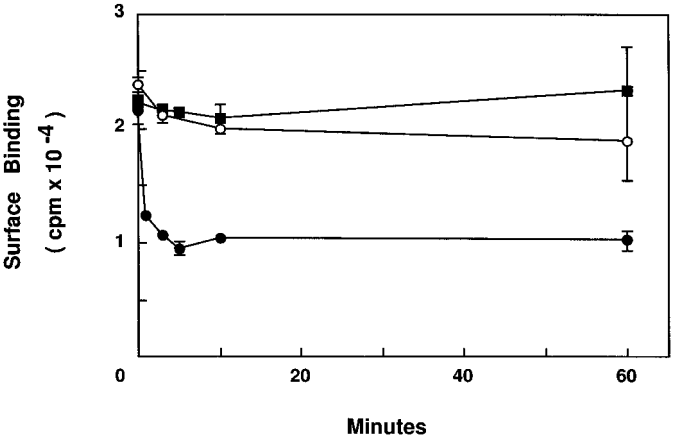


**Fig. 8.** Effect of antagonist pretreatment on cell surface receptors. HIT cells ($4 \times 10^{6}$/dish) were incubated at 37° with 100 nM concentration of either [D-Tyr$^{6}$]Bn(6–13)PA (○), PMA and [D-Tyr$^{6}$]Bn(6–13)PA (■), or Bn (●). At the indicated times, the cells were washed for 5 min at 4° with HBSS containing 20 mM glycine, pH 3.0, to remove surface-bound peptide. Subsequently, the cells were incubated at 4° in fresh buffer with [$^{125}$I-Tyr$^{4}$]Bn ($7 \times 10^{4}$ cpm/ml) in the absence or presence of 100 nM unlabeled Bn. After 4 hr, the specific binding was determined as described in Experimental Procedures.

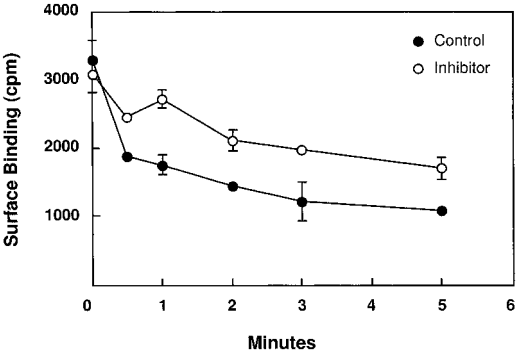


**Fig. 9.** Effect of PKC inhibition on agonist-stimulated Bn receptor internalization. HIT cells ($1.6 \times 10^{6}$/dish) were pretreated for 15 min with either 0.1% DMSO (●) or 4 $\mu$M GF109203X in 0.1% DMSO (○). After the addition of 100 nM Bn, the cells were incubated at 30° for the times shown and then washed for 5 min at 4° with HBSS containing 20 mM glycine, pH 3.0, to remove surface-bound peptide. Cell surface receptors were subsequently measured after a 90-min binding incubation at 4° with [$^{125}$I-Tyr$^{6}$]Bn(6–13)PA ($1 \times 10^{5}$ cpm/ml) in the absence or presence of 100 nM unlabeled Bn.

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caused a 50% reduction in cell surface receptors within 5 min. Together, these results demonstrate that receptor activation is required for GRP-R endocytosis.

Agonist-induced receptor activation has two distinct, albeit related, consequences: the stabilization of an activated receptor conformation by bound ligand and the stimulation of second-messenger formation with consequent PKC activation in the case of PLC-coupled receptors. PKC phosphorylation has been reported to have complex and varied effects on the internalization of GPCRs depending on both the receptor and its environment. For some PLC-coupled receptors, PKC activation seems to be directly involved in agonist-induced internalization (Liles *et al*., 1986; Fonseca *et al*., 1995; Bock *et al*., 1997). For other GPCRs, PKC activation does not affect receptor internalization (Hinkle and Shanshala, 1989). Finally, PKC activation may inhibit (Hoover and Toews, 1990) or stimulate (Signoret *et al*., 1997) agonist-induced receptor internalization. The situation for the GRP-R is unclear even though mutant and chimeric GRP-Rs have been used to address the importance of agonist-induced second-messenger formation in receptor internalization. Two mutant GRP-Rs that do not stimulate PLC on Bn binding were found to be less effectively internalized than the wild-type receptor (Benya *et al*., 1994). Similarly, a chimeric GRP-R substituted with the third cytoplasmic loop of the m3 muscarinic cholinergic receptor (BM3L) was severely impaired in both PLC activation and receptor internalization (Tseng *et al*., 1995). However, the extent to which receptor endocytosis was inhibited in mutant GRP-Rs was not clearly related either to the receptor efficacy for PLC stimulation or to the ability of the agonist occupied receptor to interact with G proteins, as deduced from the sensitivity of agonist binding to Gpp(NH)p (Benya *et al*., 1994; Tseng *et al*., 1995). For example, neither the A263E nor the R139G mutant was able to stimulate PLC, yet agonist-induced internalization of the A263E mutant was inhibited only 50%, whereas the internalization of the R139G mutant was blocked completely (Benya *et al*., 1994). Surprisingly, agonist binding was sensitive to Gpp(NH)p in both mutant receptors. Guanine nucleotide inhibition of agonist binding was reduced by 60% in the R139G mutant. However, the effect of Gpp(NH)p on the A263E mutant was indistinguishable from the wild-type receptor. The observation that PMA pretreatment stimulated the internalization of both mutant receptors supported a role for PKC stimulation, as well as an agonist-induced conformational change, in GRP-R endocytosis (Benya *et al*., 1994). Further support for PKC involvement was provided by the observation that mutation of a PKC consensus site in the carboxyl terminus of the GRP-R partially inhibited receptor-mediated internalization of agonist (Benya *et al*., 1993). However, the relative importance of an agonist-induced conformational change and PKC-catalyzed phosphorylation for the endocytosis of the wild-type receptor remains unknown. To address this issue directly, we examined the effect of PKC activation on the internalization of the antagonist/receptor complex as well as the effect of PKC blockade on the endocytosis of the agonist/receptor complex. Because PMA stimulates GRP-R phosphorylation (Kroog *et al*., 1995b; Williams *et al*., 1996) and 7-hydroxy-staurosporine inhibits this PMA stimulation (Kroog *et al*., 1995b), either a conventional cPKC or a novel nPKC isoform must catalyze this reaction (Nishizuka, 1995). HIT cells have been shown to contain $Ca^{2+}$-phospholipid-dependent protein kinases (Lord and Ashcroft, 1984), and Bn increases the membrane association of PKC in this cell line within 2 min (Regazzi *et al*., 1990). Furthermore, PMA stimulates insulin release by HIT cells (Swope and Schonbrunn, 1988), and this stimulation is completely blocked by staurosporine (Deeney *et al*., 1996). Thus, HIT cells contain the PKC isoforms that phosphorylate the GRP-R.

We show that PMA did not promote the sequestration of the antagonist/receptor complex. This observation extends previous data showing that acute PMA treatment in the absence of any ligand does not stimulate GRP-R endocytosis either in HIT cells (Swope and Schonbrunn, 1990) or in Swiss 3T3 cells (Brown *et al*., 1987). However, it remained possible that PKC activation played a role in the internalization of the agonist/receptor complex. We found that the rate but not the extent of Bn-induced GRP-R endocytosis was reduced by the PKC inhibitor GF109203X. These results demonstrate that activation of PKC is not essential for agonist-induced GRP-R endocytosis but that it increases the rate of receptor internalization.

The conclusion that PKC does not affect the extent of GRP-R internalization is not necessarily inconsistent with the study showing that mutation of a PKC phosphorylation consensus site in the GRP-R decreased the amount of receptor/ligand complex internalized at steady state (Benya *et al*., 1993); the mutation may have affected receptor phosphorylation by a different kinase, or it could have altered receptor conformation in a critical carboxyl-terminal region. The observation that TPA increased the internalization of the agonist/receptor complex for the two signaling defective GRP-R mutants (Benya *et al*., 1994) is more puzzling. Perhaps conformational changes induced by PKC phosphorylation of these mutant receptors altered their interaction with components of the endocytic machinery, an effect that either does not occur or is not as important in the case of the wild-type receptor.

Because the antagonist [D-Tyr$^6$]Bn(6–13)PA did not elicit GRP-R endocytosis and because the extent of Bn-stimulated internalization was unaffected when PKC activation was blocked, an agonist-induced conformational change must be required for receptor sequestration. Such a conformational change may have occurred even in the signaling-deficient GRP-R mutants examined previously and allowed agonist to stimulate internalization, albeit to a lesser extent than with the wild-type receptor (Benya *et al*., 1994). This hypothesis implies that the conformational change needed for receptor endocytosis differs from that needed for PLC stimulation, perhaps because different domains of the receptor are required for the molecular interactions leading to G protein coupling and internalization. Such a model would accommodate results with several GPCRs in which antagonist, as well as agonist, binding can trigger receptor endocytosis (Conchon *et al*., 1994; Roettger *et al*., 1997).

In summary, we synthesized a new high affinity antagonist and used it to show that antagonist binding does not stimulate the internalization of the GRP-R receptor even under conditions where PKC is activated. Moreover, PKC is not essential for the agonist-induced internalization of the wild-type receptor, although it does increase the endocytic rate of the agonist/receptor complex.

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