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Pharmacological profiles of two bombesin analogues in cells transfected with human neuromedin B receptors

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Abstract

We examined the effect of two des-Met-bombesin analogues, [$(CH_3)_2$CHCO-His-Trp-Ala-Val-D-Ala-His-Leu-NHCH$_3$] (ICI 216140) and [D-Phe^6,des-Met^{14}]bombesin(6–14) ethylamide (DPDM-bombesin ethylamide), on neuromedin B-induced Ca^{2+} and [^{3}H]arachidonate release in BALB 3T3 cells transfected with human neuromedin B receptors. ICI 216140 and DPDM-bombesin ethylamide both stimulated Ca^{2+} mobilisation in a concentration-dependent manner but were less potent and efficacious than neuromedin B. BIM 23042 [D-Nal-Cys-Tyr-D-Trp-Lys-Val-Cys-Nal-NH$_2$], a selective neuromedin B antagonist and [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P, a broad-spectrum peptide receptor antagonist inhibited neuromedin B-, ICI 216140- and DPDM-bombesin ethylamide-induced Ca^{2+} release. Pretreatment of cells with either des-Met-bombesin analogue attenuated neuromedin B-induced Ca^{2+} elevations, suggesting similar agonist-sensitive Ca^{2+} pools. The pharmacological profiles revealed from the [^{3}H]arachidonate assay were similar, although ICI 216140 was less potent and efficacious than DPDM-bombesin ethylamide. The data suggest that ICI 216140 and DPDM-bombesin ethylamide behave as agonists at the neuromedin B receptor, perhaps as a consequence of neuromedin B receptor overexpression.

Keywords: Neuromedin B; Bombesin receptor antagonist; Ca^{2+}, intracellular; Arachidonic acid

1. Introduction

Neuromedin B, a mammalian peptide of the bombesin-like peptide family sharing amino acid homology with its amphibian counterpart ranatensin, elicits a diverse array of biological responses in central and peripheral tissues. Pharmacological evidence (Von Schrenck et al., 1989, 1990; Ladenheim et al., 1990) and data from receptor cloning studies (Spindel et al., 1990; Corjay et al., 1991; Wada et al., 1991; Gorbulev et al., 1992; Fathi et al., 1993) strongly support the existence of three receptor subtypes in mammals: a neuromedin B-preferring receptor (BB_1), a gastrin releasing peptide-preferring receptor (BB_2) and a third receptor (BB_3) which binds poorly to bombesin or neuromedin B. A fourth subtype (BB_4), selective for [Phe^{13}]bombesin, was cloned recently and characterised from the amphibian *Bombina orientalis* (Nagalla et al., 1995). For all bombesin-like peptide receptors studied so far, analysis of their amino acid sequences reveals a motif characteristic of the G-protein-coupled receptor superfamily. In C_6 glioma cells (Wang et al., 1992), NCI-H345 small cell lung carcinoma cells (Moody et al., 1992) and BB_1-transfected BALB fibroblasts (Benya et al., 1992, 1995; Dobrzanski et al., 1993), binding of neuromedin B to the BB_1 receptor activates phospholipase C, generating inositol metabolites and stimulating Ca^{2+} release.

Of particular interest is the observation that bombesin-like peptides stimulate Ca^{2+} mobilisation and growth in a variety of tumors. Several workers have demonstrated inhibition of bombesin-induced signaling or growth by BB_2 receptor antagonists in tumors of breast (Patel and Schrey, 1992; Yano et al., 1992, 1994; Shirahige et al., 1994), colon (Frucht et al., 1992; Radulovic et al., 1992), prostate (Pinski et al., 1993; Reile et al., 1994), stomach (Halmos et al., 1994; Pinski et al., 1994; Qin et al., 1994) and small cell lung carcinoma (Mahmoud et al., 1989, 1991; Staley et al., 1991; Bunn et al., 1994). We have reported that two BB_2 receptor antagonists, the des-Met-bombesin analogues ICI 216140 (Camble et al., 1989) and DPDM-bombesin ethylamide (Wang et al., 1990), non-competitively inhibited neuromedin B-induced Ca^{2+} flux

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in the small cell lung carcinoma line NCI-H345, suggesting that these compounds also interact with BB_1 receptors (Ryan et al., 1993). Because NCI-H345 cells contain binding sites for gastrin releasing peptide and neuromedin B, delineating the contribution of the gastrin releasing peptide receptor to neuromedin B-induced Ca^{2+} release becomes a complex issue. Homologous populations of BB_1 binding sites have been described on small cell lung carcinoma lines (Moody et al., 1992), but their low level of receptor expression makes meaningful Ca^{2+} measurement difficult. We therefore investigated the effects of bombesin-like peptides and these BB_2 receptor antagonists on Ca^{2+} flux in BALB 3T3 fibroblasts transfected with cDNA for human bombesin-like peptide receptors (Benya et al., 1995), and compared them to the effects observed with rat C_6 glioma cells, which contain only BB_1 receptors (Wang et al., 1992).

In addition to the signaling sequelae mentioned above, bombesin elicits arachidonate formation and metabolism in Swiss 3T3 cells, which contain BB_2 receptors (Millar and Rozengurt, 1990). Likewise, fibroblasts transfected with the rodent BB_1 receptor release arachidonate when exposed to neuromedin B (Dobrzanski et al., 1993). We therefore examined, additionally, the effect of neuromedin B and the des-Met-bombesin analogues on [^{3}H]arachidonate release in human BB_1 receptor-transfected cells.

Recently, Orbuch et al. (1993) reported that certain substituted somatostatin analogues can function as selective BB_1 receptor antagonists. One analogue, BIM 23042 [D-Nal-Cys-Tyr-D-Trp-Lys-Val-Cys-Nal-NH_2], has a 100-fold greater affinity for BB_1 receptors than BB_2 receptors. Also, the substance P analogue [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P can act as a broad-spectrum peptide receptor antagonist (Woll and Rozengurt, 1988a). In the present study, we evaluated the effect of BIM 23042 and [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P on Ca^{2+} flux stimulated by the two des-Met-bombesin analogues and characterised the pharmacological profile of BIM 23042 against neuromedin B-induced [^{3}H]arachidonate release in fibroblasts transfected with the human neuromedin B receptor.

2. Materials and methods

2.1. Cell culture

C_6 glioma cells and BALB 3T3 B1 fibroblasts were grown and maintained in Dulbecco's modified eagle medium (DMEM) containing 10% fetal calf serum, 200 U/ml penicillin G and 200 μg/ml streptomycin. BALB 3T3 B1 fibroblasts transfected with human neuromedin B receptor (huNMBR cells) or human gastrin releasing peptide receptor cDNA were grown and maintained in similar media supplemented with 300 μg/ml geneticin (Life Technologies, Grand Island, NY). All cells were incubated under standard culture conditions (37°C, 5% CO_2/air mixture, 100% humidity).

2.2. Measurement of $[Ca^{2+}]$

Cells were harvested using a dissociation buffer (137 mM sodium chloride, 5 mM potassium chloride, 0.17 mM dibasic sodium phosphate, 0.22 mM potassium dihydrogen phosphate, 6 mM glucose, 59 mM sucrose; pH 7.0) and washed with serum-free, phenol red-free DMEM assay buffer (pH 7.4) containing 1 mg/ml bovine serum albumin. After determining cell number and viability via trypan blue extrusion, 2.5 μM fura-2-acetoxymethyl ester was added, incubated for 45 min at 37°C and allowed to sit at room temperature for 15 min. The cells were centrifuged ($1000 \times g$, 2 min), washed twice with assay buffer and resuspended to a final concentration of 3×10^5 cells/ml. For the assay, a 1 ml sample of cell suspension was placed in a cuvette and put into a Perkin Elmer LS-5 fluorimeter equipped with a stir bar and water bath (37°C). The change in $[Ca^{2+}]$ as a function of fluorescence (excitation wavelength = 340 nm, emission wavelength = 510 nm) was measured in the presence of neuromedin B, DPDM-bombesin ethylamide, ICI 216140, [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P or BIM 23042. Calculation of $[Ca^{2+}]$ was conducted as described by Grynkiewicz et al. (1985).

2.3. [^{3}H]Arachidonate release

The protocol employed was a slightly modified version of the method utilised for BALB 3T3 cells transfected with rodent neuromedin B receptors (Dobrzanski et al., 1993). huNMBR cells were grown in 24-well plates and allowed to reach confluence. The cells were washed with serum-free, phenol red-free DMEM (pH 7.4) containing 1 mg/ml fatty acid-free bovine serum albumin and incubated in this buffer containing 2.5 μCi/ml [^{3}H]arachidonate (Amersham) for 4 h at 37°C. The wells were washed twice and 1 ml DMEM containing the peptide(s) of interest was added. After a 30 min incubation at 37°C, a 900 μl aliquot of media was removed and centrifuged. A portion of the supernatant (750 μl) was mixed with Ecoscint scintillation cocktail and radioactivity measured in a scintillation counter.

2.4. Materials

Bombesin, gastrin releasing peptide, neuromedin B, [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P and DPDM-bombesin ethylamide were purchased from Peninsula Labs. BIM 23042 was generously supplied by Biomeasure, Milford, MA. ICI 216140 was purchased from Cambridge Research Biochemicals, Wilmington, DE. DMEM was obtained from Sigma. All other chemicals were reagent grade.

2.5. Data analysis

Data plotting and iterative curve fitting were performed using Kaleidagraph graphing software (Synergy Software, Reading, PA).

3. Results

3.1. Cell Ca^{2+}

Neuromedin B stimulated Ca^{2+} mobilisation in a concentration-dependent manner in C_6 cells (Fig. 1) with an EC_{50} of 3.6 ± 0.7 nM. Both ICI 216140 and DPDM-bombesin ethylamide failed to mobilize Ca^{2+} on their own, and attenuated the neuromedin B-induced Ca^{2+} flux in a competitive manner. DPDM-bombesin ethylamide was a more effective antagonist ($K_i = 498 \pm 7$ nM) than ICI 216140 ($K_i = 3.8 \pm 0.15$ μM) against neuromedin B.

In fibroblasts transfected with human BB_1 or BB_2 receptors, bombesin-like peptides stimulated Ca^{2+} flux in a concentration-dependent manner (Fig. 2). Bombesin was 10-fold more potent than neuromedin B in cells transfected with gastrin releasing peptide receptors. EC_{50} values of 2.9 ± 0.8 nM and 29 ± 15 nM, respectively, were obtained. Gastrin releasing peptide was slightly less potent (14 ± 5.3 nM), but more efficacious, than bombesin. Neuromedin B was more potent (0.9 ± 0.2 nM) than either bombesin (2.5 ± 0.8 nM) or gastrin releasing peptide (55 ± 27 nM) in huNMBR cells. ICI 216140 and DPDM-bombesin ethylamide stimulated Ca^{2+} mobilisation but were less potent and efficacious than neuromedin B (Fig. 3) with an EC_{50} of 896 ± 543 nM and 121 ± 36 nM, respectively. Neither antagonist studied elevated Ca^{2+} in untransfected BALB 3T3 cells nor in cells containing human gastrin releasing peptide receptors ($n = 6$). In the huNMBR cells, pretreatment with either des-Met-bombesin analogue attenuated the Ca^{2+} response to a submaximal concentration of neuromedin B (1 nM) in a concentration-dependent manner (Fig. 4). BIM 23042 (5 μM) was more effective than [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P (5 μM) in attenuating either DPDM-bombesin ethylamide or ICI 216140-induced Ca^{2+} flux (Fig. 5). The submaximal mobilisation observed with neuromedin B (1 nM) was abolished by BIM 23042 (5 μM) but restored with a subsequently higher concentration of neuromedin B (1 μM) (Fig. 5).

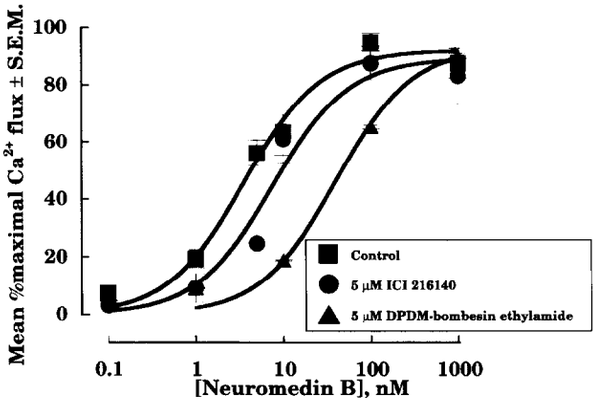

Fig. 1. Effect of the des-Met-bombesin antagonists DPDM-bombesin ethylamide and ICI 216140 on neuromedin B-induced Ca^{2+} mobilization in C_6 cells. The change in [Ca^{2+}] from basal levels to the peak of the transient after neuromedin B stimulation was monitored in the presence and absence of 5 μM ICI 216140 or 5 μM DPDM-bombesin ethylamide at the concentrations indicated. The data represent the percent of maximal response attained at a concentration of 1000 nM neuromedin B. The plot represents means ± S.E. mean taken from at least three separate experiments ($n = 3$–6). The basal Ca^{2+} concentration was 75 ± 5.8 nM, and the value after 1 μM of neuromedin B was 209 ± 24 nM.

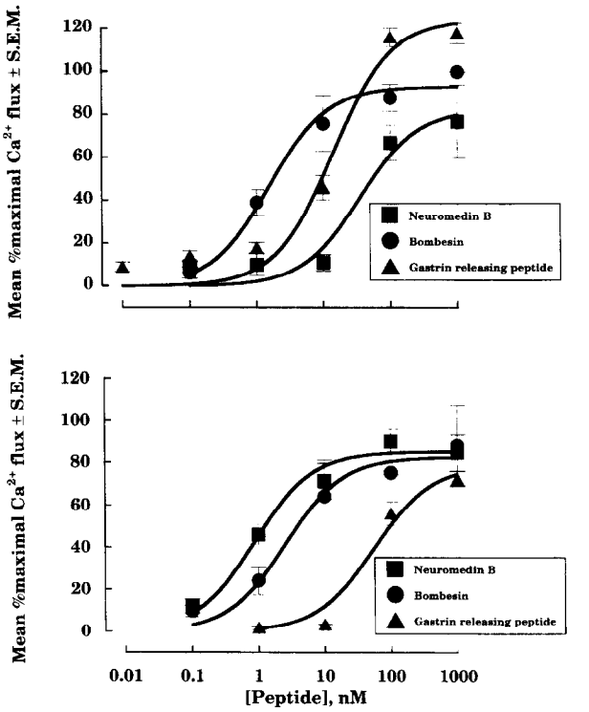

Fig. 2. Effect of bombesin-like peptides on Ca^{2+} mobilisation in transfected BALB 3T3 cells. Upper panel: the change in [Ca^{2+}] from basal levels to the peak of the transient was monitored in the presence of bombesin, gastrin releasing peptide or neuromedin B at the concentrations indicated in human gastrin releasing peptide receptor-transfected cells. The data represent the percent of response attained at a concentration of 1 μM bombesin and are the means ± S.E. from four separate preparations. The basal Ca^{2+} concentration was 79 ± 13 nM, and the value after 1 μM of bombesin was 820 ± 123 nM. Lower panel: in huNMBR cells, the change in [Ca^{2+}] from basal levels to the peak of the transient was monitored in the presence of bombesin, gastrin releasing peptide or neuromedin B at the concentrations indicated. Data are represented as the percent of response attained at a concentration of 1 μM neuromedin B, and are the means ± S.E. from six separate preparations. The basal Ca^{2+} concentration was 98 ± 9.6 nM, and the value after 1 μM of neuromedin B was 553 ± 54 nM.

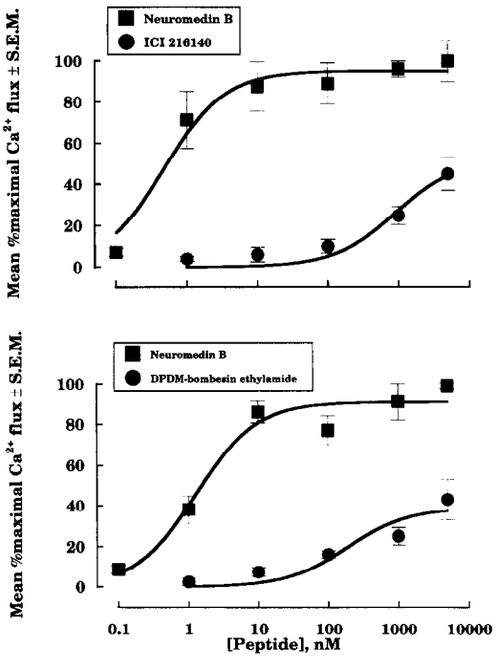

Fig. 3. Effect of des-Met-bombesin analogues on Ca^{2+} mobilisation in huNMBR cells. The change in $[Ca^{2+}]$ from basal levels to the peak of the transient after ICI 216140 (upper panel) or DPDM-bombesin ethylamide (lower panel) stimulation was recorded at the concentrations indicated. The data represent the percent of maximal response attained at a concentration of 5 μM neuromedin B. The upper panel represents 4–7 determinations taken from three cell preparations. The lower panel represents the means ± S.E. from six cell preparations. The mean basal Ca^{2+} concentration was 60 ± 13 nM, and the value after 5 μM of neuromedin B was 656 ± 166 nM.

3.2. [^{3}H]Arachidonate studies

In the [^{3}H]arachidonate assay, neuromedin B elicited concentration-dependent elevations of [^{3}H]arachidonate-associated radioactivity in supernatants of huNMBR cells with an EC_{50} of 9.5 ± 1.9 nM. DPDM-bombesin ethylamide was less potent (EC_{50} = 220 ± 79 nM) and efficacious than neuromedin B (Fig. 6). Notably, the only statistically significant elevation of radioactivity observed with ICI 216140 was at a concentration of 10 μM (Dunnett's t test, two-tailed, $P < 0.05$). BIM 23042 (5 μM) competitively inhibited this neuromedin B-induced endpoint in huNMBR cells with a K_i of 49 ± 14 nM (Fig. 7). Since DPDM-bombesin ethylamide was more potent and efficacious than ICI 216140 in this assay, we examined the effect of BIM 23042 on only DPDM-bombesin ethylamide-induced [^{3}H]arachidonate release, and found that BIM 23042 was a potent inhibitor of this des-Met analogue (Fig. 8).

4. Discussion

In a previous study, we demonstrated that two des-Met-bombesin analogues, ICI 216140 and DPDM-bombesin ethylamide, inhibited neuromedin B-induced Ca^{2+} flux in a noncompetitive manner in NCI-H345 cells (Ryan et al., 1993). However, Moody et al. (1985, 1992) had reported that neuromedin B could, at sufficiently high concentrations, interact with the BB_2 receptor in this cell line. Furthermore, it was possible both des-Met-bombesin analogues behaved as partial agonists on BB_1 receptors with a low intrinsic activity beyond the limits of assay sensitivity. In this study, we examined the pharmacological profiles of bombesin-like peptides and these bombesin antagonists against neuromedin B-induced Ca^{2+} mobilisation in C_6 and huNMBR cells and compared them with those observed in our previous work with NCI-H345 cells. We found that bombesin, gastrin releasing peptide and neuromedin B mobilised Ca^{2+} in cells transfected with

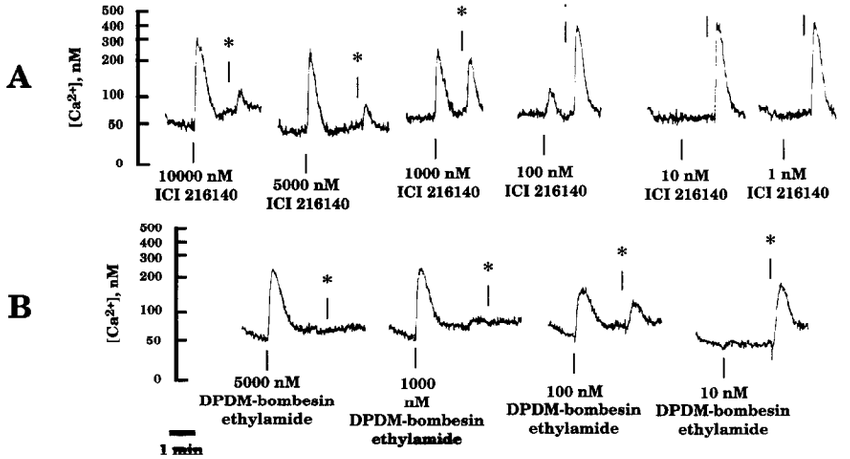

Fig. 4. Desensitisation of neuromedin B-induced Ca^{2+} mobilisation by des-Met-bombesin analogues in huNMBR cells. While recording fura-2 fluorescence, cells were pretreated with ICI 216140 (A) or DPDM-bombesin ethylamide (B) at the concentrations indicated and allowed to return to steady-state Ca^{2+} levels. Neuromedin B (100 nM) was then added (*). Results are from a typical experiment performed at least three times.

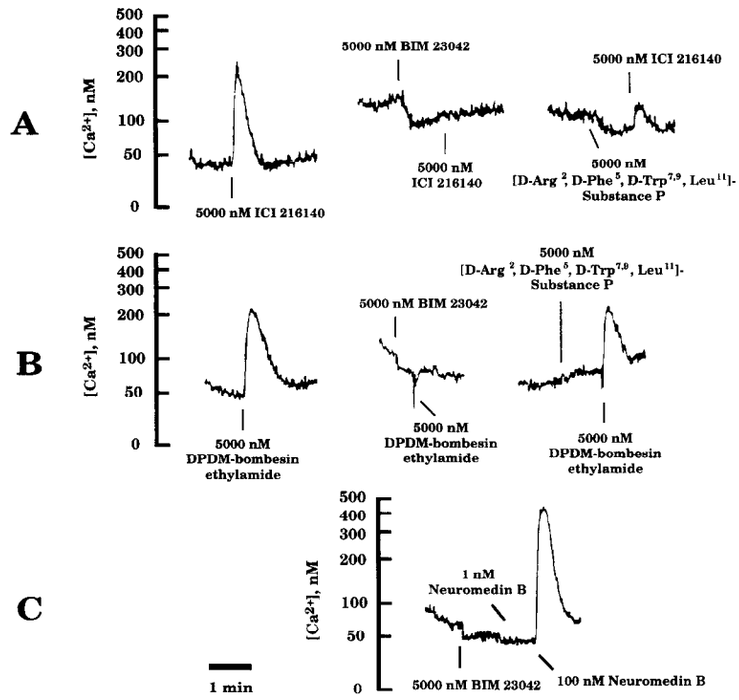

Fig. 5. Effect of bombesin-like peptides and antagonists on Ca^{2+} flux in huNMBR cells. Cells were incubated in the presence or absence (control) of 5 μM BIM 23042 or [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P for 1 min, after which ICI 216140 (A), DPDM-bombesin ethylamide (B) or neuromedin B (C) was added at the concentrations indicated. Results are representative of a typical experiment performed three times.

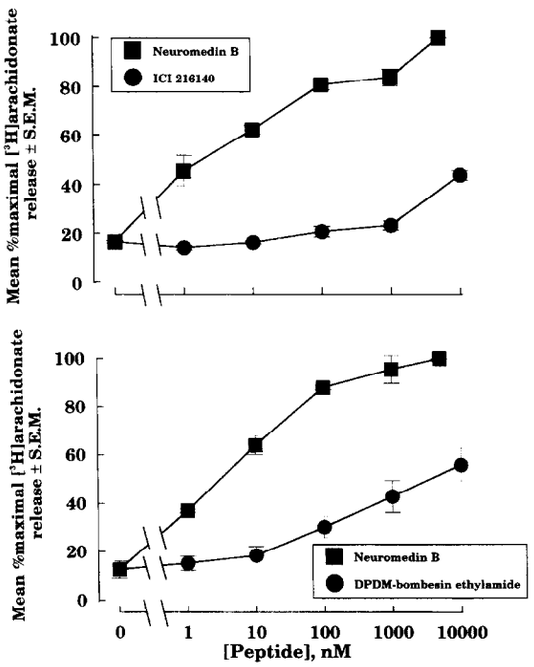

Fig. 6. Effect of des-Met-bombesin analogues on [^{3}H]arachidonate release in huNMBR cells. Cells were assayed as described in the Methods in the presence of either neuromedin B, ICI 216140 (upper panel) or DPDM-bombesin ethylamide (lower panel). Values represent the percent maximal response attained at a concentration of 5 μM neuromedin B and are the means ± S.E. from three separate experiments performed in duplicate. Standard error values smaller than the symbol size are enclosed within the symbol.

bombesin-like peptide receptors, and their selectivity for these peptides correlated well with what one would expect in cells expressing only gastrin releasing peptide- or neuromedin B-preferring binding sites (Benya et al., 1995). In contrast to the NCI-H345 cells, we found that both ICI 216140 and DPDM-bombesin ethylamide behaved as competitive antagonists in C_6 cells yet stimulated Ca^{2+} flux in a concentration-dependent manner in the huNMBR cells. Since untransfected BALB 3T3 cells and BB_2 receptor-transfected cells failed to mobilise Ca^{2+} in response to

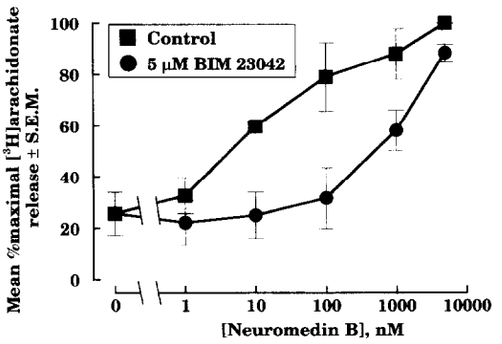

Fig. 7. Effect of BIM 23042 on neuromedin B-induced [^{3}H]arachidonate release in huNMBR cells. huNMBR cells were assayed in the presence or absence (control) of 5 μM BIM 23042. Values represent the percent maximal response attained at a concentration of 5 μM neuromedin B and are the means ± S.E. from three separate experiments performed in duplicate.

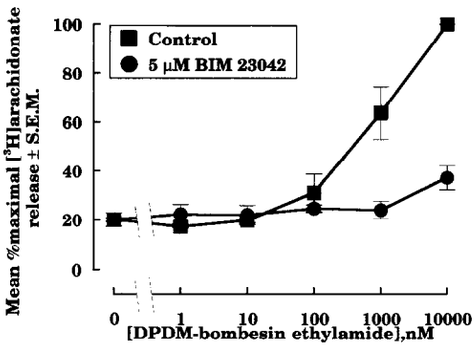

Fig. 8. Effect of BIM 23042 on DPDM-bombesin ethylamide-induced [^{3}H]arachidonate release in huNMBR cells. huNMBR cells were assayed in the presence or absence (control) of 5 μM BIM 23042. Values represent the percent maximal response attained at a concentration of 5 μM DPDM-bombesin ethylamide and are the means ± S.E. from three separate experiments performed in duplicate.

these two des-Met-bombesin analogues, it is unlikely that ICI 216140 and DPDM-bombesin ethylamide interacted with any endogenous receptor(s) in the huNMR cells. In addition, pretreatment with either analogue attenuated neuromedin B-stimulated Ca^{2+} flux, suggesting that these compounds used the same agonist-induced Ca^{2+} pools utilised by BB_1 receptors. [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]-substance P, a broad-spectrum peptide receptor antagonist (Woll and Rozengurt, 1988a,b), also attenuated neuromedin B-, DPDM-bombesin ethylamide- and ICI 216140-induced Ca^{2+} flux. The most convincing evidence was the observation that BIM 23042, a selective BB_1 receptor antagonist, blocked the effects of both DPDM-bombesin ethylamide and ICI 216140.

We also examined the effects of neuromedin B, DPDM-bombesin ethylamide and ICI 216140 on [^{3}H]arachidonate mobilisation in huNMBR cells. The profiles obtained with these peptides were similar to those noted in the Ca^{2+} studies, including the observation that ICI 216140 was a very weak agonist. A major structural difference between ICI 216140 and DPDM-bombesin ethylamide is that the former contains an isobutryl group at the N-terminus portion of the peptide (Camble et al., 1989); perhaps this structural difference accounts for the results.

We are first to report the effects of BIM 23042 on neuromedin B-induced Ca^{2+} mobilisation and neuromedin B-induced [^{3}H]arachidonate release in huNMBR-transfected cells. Consonant with other studies (Orbuch et al., 1993; Benya et al., 1995), BIM 23042 was a potent competitive antagonist in these functional assays. In addition, BIM 23042 was a potent inhibitor against the elevation of [^{3}H]arachidonate-associated radioactivity stimulated by DPDM-bombesin ethylamide. It remains to be determined whether or not the radioactivity from supernatants collected from the [^{3}H]arachidonate assay represented arachidonate only, eicosanoid only, or both.

We are currently repeating these studies with BALB 3T3 fibroblasts transfected with the rat esophageal neuromedin B receptor (Dobrzanski et al., 1993), and the preliminary data appear similar to those observed with huNMBR cells (unpublished results). The amino acid sequence between these two species varies by 10% (Corjay et al., 1991), yet it seems unlikely that the difference in primary structure between rodent and human BB_1 receptors could account for the newly characterised pharmacological profiles of the des-Met-bombesin analogues.

The data may point towards a pharmacodynamic mechanism for the interaction of ICI 216140 and DPDM bombesin-ethylamide against the BB_1 receptor in NCI-H345 cells. Previous binding studies in this cell line revealed 1500 neuromedin B-binding sites/cell (Moody et al., 1992). The C_6 cells and huNMBR cells have been found to contain at least 10- and 20-fold more neuromedin B-binding sites/cell, respectively (R.T. Jensen, personal communication), so that the effects observed with these bombesin analogues may be a function of receptor number. Because NCI-H345 cells contain far fewer BB_1 sites, we were previously unable to detect any agonist activity using the Ca^{2+} assay (Ryan et al., 1993). The Ca^{2+} mobilisation assay continues to be a valuable investigative tool, but the data illustrate the importance of appreciating receptor number before reaching conclusions regarding pharmacodynamic mechanisms of bombesin-like peptides and/or their antagonists. Wu et al. (1995) recently reported that in BALB 3T3 cells transfected with human gastrin releasing peptide receptors, DPDM-bombesin ethylamide stimulated DNA synthesis yet failed to stimulate Ca^{2+} flux. However, when these cells expressed 30-fold greater levels of gastrin releasing peptide-specific binding sites, DPDM bombesin-ethylamide behaved as a partial agonist in the Ca^{2+} mobilisation response.

In conclusion, we have found that ICI 216140 and DPDM-bombesin ethylamide behave as neuromedin B receptor agonists in human neuromedin B receptor-transfected BALB 3T3 fibroblasts, and that their pharmacological profile may be a consequence of non-native BB_1 receptor overexpression.

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