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Identification and characterisation of functional bombesin receptors in human astrocytes

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Abstract

Reverse transcription polymerase chain reaction (RT-PCR) demonstrated the presence of bombesin BB2 receptor mRNA but not bombesin BB1 receptor or bombesin BB3 receptor mRNA in cultured human astrocytes. Neuromedin C hyperpolarised human astrocytes in whole-cell current and voltage clamp recordings and increased the intracellular free Ca^{2+} ion concentration ($[Ca^{2+}]_i$) in single astrocytes. Treatment with neuromedin C caused larger and more frequent increases in $[Ca^{2+}]_i$ than those triggered by neuromedin B, with 96% and 78% of cells responding, respectively. The stimulatory effects of neuromedin C were inhibited significantly by treatment with U73122 or the bombesin BB2 receptor antagonist [D-Phe^6, des-Met^{14}]bombesin-(6–14) ethylester. A Fluorometric Imaging Plate Reader (FLIPR) was used to measure $[Ca^{2+}]_i$ in cell populations. Neuromedin C was approximately 50-fold more potent than neuromedin B in elevating $[Ca^{2+}]_i$ in astrocytes and Chinese hamster ovary (CHO) cells expressing human bombesin BB2 receptors (hBB2-CHO). However, in CHO cells expressing the bombesin BB1 receptor hBB1-CHO, neuromedin B was 32-fold more potent than neuromedin C. [D-Phe^6, des-Met^{14}]bombesin-(6–14) ethylester was a partial agonist in hBB1-CHO cells (E_{max} = 55%) but was a noncompetitive antagonist in both hBB2-CHO cells and astrocytes. These studies report the first identification of functional bombesin receptors on cultured human astrocytes and have demonstrated that the bombesin BB2 receptor contributes significantly to astrocyte physiology. © 2002 Published by Elsevier Science B.V.

Keywords: Astrocyte; CHO cell; FLIPR; Ca^{2+}; Bombesin; [D-Phe^6, des-Met^{14}]bombesin-(6–14) ethylester

1. Introduction

Bombesin was originally isolated from frog skin (Anastasi et al., 1971). Recently, three mammalian counterparts, neuromedin B, a gastrin-releasing peptide, and neuromedin C, the biologically active terminal gastrin-releasing peptide fragment, have been discovered. These peptides and their high-affinity binding sites are widely distributed throughout the mammalian central nervous system (CNS) (Ladenheim et al., 1990; Moody et al., 1981; Zarbin et al., 1985). At least in the rat, studies have shown that bombesin receptors are expressed on astrocytes, as well as on neurones (Hösli et al., 1992; Hösli and Hösli, 1993a,b, 1994). Bombesin receptors located on rat astrocytes are reported both to mediate membrane hyperpolarization and to couple to increased levels of intracellular free calcium ($[Ca^{2+}]_i$) (Hösli et al., 1993; Enkvist et al., 1989). However, little is known about the pharmacology of the receptor subtypes present or of the functional significance of their activation on astrocytes. Moreover, functional bombesin receptors have not yet been identified on primary human astrocytes. Pharmacological characterisation of the receptors present on human astrocytes has been undertaken in part by using recombinant receptors expressed in Chinese hamster ovary (CHO) host cells as a model system for comparative studies.

To date, three mammalian bombesin receptors have been cloned: the bombesin BB1 (neuromedin B-preferring), BB2 (gastrin-releasing peptide preferring), and BB3 subtypes (Wada et al., 1991; Spindel et al., 1990; Fathi et al., 1993). All three couple via G_q to the phospholipase C signaling pathway, generating inositol lipid metabolites and releasing Ca^{2+} from intracellular stores (Wang et al., 1992; Moody et al., 1992; Sharif et al., 1997; Wu et al., 1996). In the present study, we have employed Ca^{2+} imaging, electrophysiolog-

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ical, molecular biological, and radioligand binding techniques to characterise the bombesin receptors present on human astrocytes. In addition to the appropriate agonists, we have used the selective bombesin BB2 receptor antagonist, [d-Phe^{6}, des-Met^{14}]bombesin-(6–14) ethylester, (Wang et al., 1990) to define the pharmacology of responses. Wherever possible, CHO cells stably transfected with human bombesin BB1 or BB2 receptors have been used alongside primary human astrocytes, providing direct comparisons of receptor characteristics.

2. Methods

2.1. Cell culture

2.1.1. CHO cells

Chinese hamster ovary (CHO) cells stably transfected with the human bombesin BB1 (hBB1-CHO) and human bombesin BB2 (hBB2-CHO) receptors were maintained in medium (nutrient mixture F-12 (Ham) + glutamax-1) containing 10% foetal bovine serum in the presence of 100 μg/ml G418. Cells were harvested by washing in warmed phosphate-buffered saline followed by a 2–3 min incubation with trypsin–EDTA solution [0.5% porcine trypsin and 0.02% EDTA·4Na in Hanks Buffered Salt Solution (HBSS)] at 37 °C. Detached cells were centrifuged in their culture media for 5 min at 200 × *g* before resuspension in the appropriate volume of medium/buffer for individual assays. Cells were passaged every 5 days, a maximum of 25 times.

2.1.2. Human astrocytes

Primary human astrocytes were obtained and cultured as directed (BioWhitaker, Bethesda, MD, USA). Cells were passaged a maximum of three times in order to maintain response integrity. Cultures were detached by a prewash in and by a 5-min incubation with prewarmed trypsin–EDTA solution (0.5% porcine trypsin and 0.02% EDTA·4Na in HBSS). When approximately 50% of the cells were dissociated, these cells were transferred to trypsin-free medium. To detach the remaining cells, fresh trypsin–EDTA solution was added for a further 3–5 min. Except for differences in media, harvested astrocytes were treated using the same protocols as used for CHO cell lines.

2.2. Immunolabeling

Immunolabeling of cells with glial fibrillary acidic protein (GFAP) was undertaken using protocols described previously (Rossant et al., 1999). Briefly, mouse monoclonal GFAP-specific antibodies were obtained from Boehringer Mannheim and used at a concentration of 1:1000. The presence of primary antibody was confirmed using a fluorescein-tagged anti-mouse secondary antisera (Vector Laboratories). No staining was apparent when primary antibodies were excluded from the immunolabeling protocol. Cells were visualised and imaged using a Leica confocal microscope system and software.

2.3. Single-cell analysis of intracellular free Ca^{2+}

For single-cell imaging experiments, cells were grown in their relevant culture media on 22 mm coverslips overnight and loaded with 2 μM Fura-2-AM in physiological saline containing (in mM) 135.0 NaCl, 5.0 KCl, 1.0 $MgCl_2$, 1.0 $CaCl_2$, 10.0 HEPES, and 3.0 glucose at pH 7.3 for 1 h at room temperature. After loading, coverslips were mounted into imaging chambers and perfused with extracellular medium to remove extracellular Fura-2-AM and to enable hydrolysis of intracellular Fura-2-AM to occur. Measurements of changes in $[Ca^{2+}]_i$ in individual cells were determined following sequential excitation of cells at 340 and 380 nm with analysis of fluorescence emission at > 510 nm using a spectral Wizard monochromator, cooled integrating CCD camera and a dedicated suite of software (Merlin, Life Sciences Resources, Cambridge, UK). Data are expressed in ratio units (RU) where RU=(emission intensity at 510 nm following excitation at 340 nm)/(emission intensity at 510 nm following excitation at 380 nm) for sequential excitations.

2.4. Electrophysiological investigation of the effects of bombesin receptor agonists on human astrocytes

The whole-cell configuration of patch clamp technique was employed. Cells were visually identified and bathed at room temperature in saline solution containing (in mM) 140 NaCl, 5.0 KCl, 1.0 $CaCl_2$, 1.0 $MgCl_2$, and 10.0 HEPES at pH 7.2, while the intracellular (pipette) solution comprised (in mM) 120.0 Kgluconate, 10.0 NaCl, 2.0 $MgCl_2$, 0.5 K_2EGTA, 10.0 HEPES, 4.0 Na_2ATP, 0.1 Na_2GTP (pH 7.2). In whole-cell voltage clamp experiments, cells were held at − 60 mV and depolarising ramps (− 140 to − 60, 20 mV/s) were evolved to assess reversal potential.

2.5. Population analysis of intracellular free Ca^{2+} changes using Fluorometric Imaging Plate Reader (FLIPR)

2.5.1. CHO cells

For the study of $[Ca^{2+}]_i$ in cell populations, cells were seeded (30,000/well) the previous day in black-walled, clear-based 96-well plates (Costar UK). After 24 h, growth medium was removed and the cells were washed three times in freshly prepared Hanks buffered salt solution (HBSS), containing 20 mM HEPES and 2.5 mM probenicid (pH 7.4 at room temperature) using a Labsystems cell washer. Cells were loaded in the same buffer (total volume = 200 μl) containing 1 μM Fluo-4-AM dye (premixed with an equal volume of 20% pluronic F-127 solution) for 1 h (37 °C) in a 5% CO_2 incubator at 95% humidity. Washing was repeated to remove extracellular dye and cells were incubated (20 min at 37 °C) in the absence/presence of 50 μl antagonist

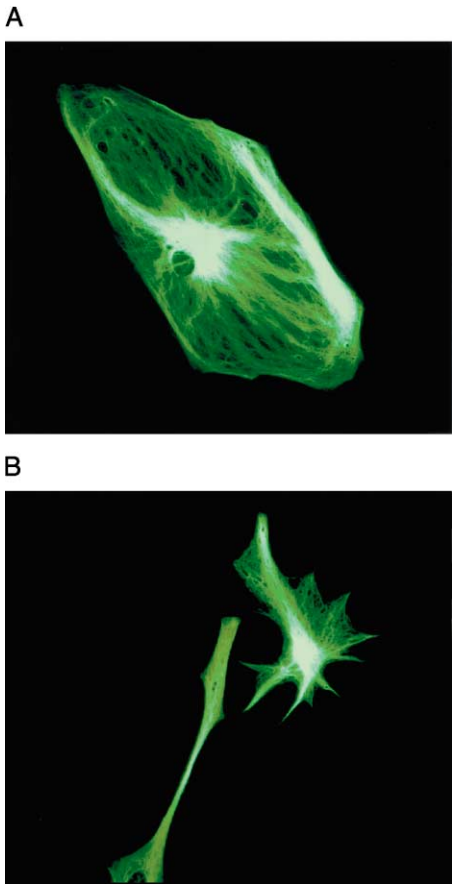

Fig. 1. Representative confocal images are presented of three primary human astrocytes imaged after 2 days in culture. Monoclonal antibodies against the astrocyte marker glial fibrillary acidic protein were used to characterise cultured cells. Cells were labelled using a fluorescein-tagged secondary antibody and visualised at 100× magnification using a Leica confocal system.

solution (total volume = 150 μl) before transfer to the FLIPR drawer. Laser intensity was set (0.2–0.5 W) to provide a basal fluorescence signal of 10,000 relative fluorescence units (RFU) and measurements of fluorescence were taken at 1-s intervals for the first 60 s, then at 10-s intervals for the remaining 60 s. An on-board 96-well pipettor allowed simultaneous addition of agonist doses (50 μl) delivered at a rate of 60 μl/s, 10 s after the start of readings. Fluorescence measurements were captured by a cooled CCD camera and integrated to an on-line PC. Data were exported as the maximum peak height change in fluorescence.

2.5.2. Astrocytes

For these cells, the same protocol was used with minor modifications. Cells were seeded 24 h prior to experimentation at 15,000 cells/well and washed in HBSS buffer excluding probenicid. Cells were loaded with Fluo-3-AM dye (4 μM) and agonists were added at a reduced speed of 40 μl/s. All concentration–response curves for the agonists were analysed using the computerised curve-fitting package, GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego, CA, USA). Values for EC_{50}, slope factor ("Hill coefficient") and maximum asymptote were determined by fitting data to a sigmoidal (variable slope) nonlinear regression curve-fitting response against log (concentration).

Noncompetitive antagonist affinities were estimated using a method first used by Gaddum et al. (1955) and described by Kenakin (1997). This method employed plotting the reciprocals of equi-active concentrations of agonist in the absence and presence of antagonist. By applying the following equation to the resultant straight-line plot, the affinity (K_B) of the antagonist (B) was determined:

$$K_B = [B]/(\text{slope} - 1).$$

Similarly, using the same reciprocal regression plot, the agonist affinity (K_A) was also calculated as follows:

$$K_A = (\text{slope} - 1)/\text{intercept}.$$

2.6. [^{125}I]Bombesin radioligand binding

CHO cells or human astrocytes were harvested as described and homogenised in ice-cold Tris–HCl buffer (pH 7.4) using a glass/Teflon homogeniser. Membranes were centrifuged twice for 10 min at 48,000 × g at 4 °C, with an intermediate resuspension of the first pellet in fresh buffer. Final pellets were homogenised in fresh Tris–HCl to give a membrane concentration equivalent to 0.8 (CHO) and 0.4 (astrocytes) million cells/ml, and kept on ice until ready for assay.

For the binding assay, 125 μl of cell homogenate was added to appropriate concentrations of [^{125}I]bombesin, displacing drugs and buffer to give a final incubation volume of 250 μl. Peptidase inhibitors (2 μM chymostatin and phosphoramidin; 4 μM leupeptin; 40 μM bacitracin) and 0.02% bovine serum albumin were present in all incubations, and nonspecific binding was defined by 1 μM bombesin. Incubations (25 °C for 1 h) were terminated by rapid filtration through GF/B filters (presoaked in 0.2% polyethylenimine)

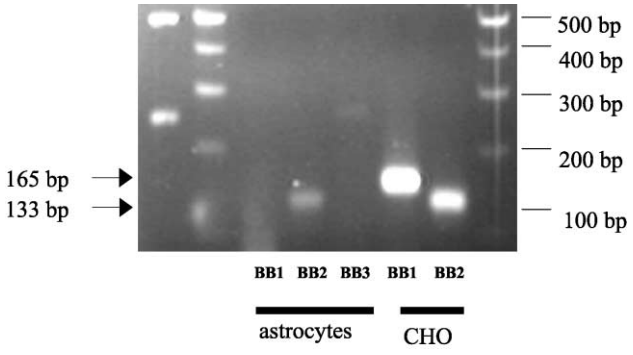

Fig. 2. Typical example of reverse transcription polymerase chain reaction analysis of human bombesin receptor gene expression in cultured primary human astrocytes and CHO cells expressing either human bombesin BB1 (BB1) receptor or human BB2 (BB2) receptor (n = 3). PCR products of the expected sizes are shown: BB1—165 bp; BB2—133 bp; BB3—184 bp.

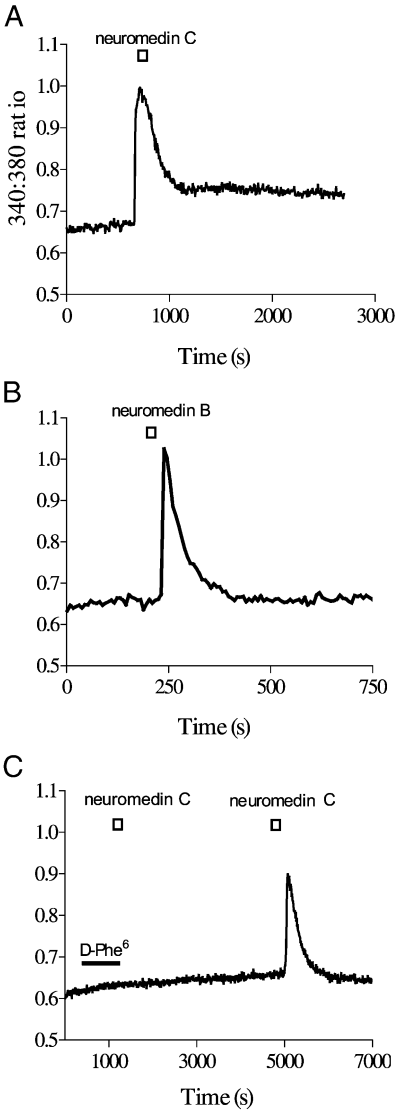

Fig. 3. Single-cell Ca^{2+} analysis. Representative traces obtained from single cells showing the effect of neuromedin C (NMC, 10 nM) and neuromedin B (NMB, 10 nM) in the absence (A,B) or, for neuromedin C, in the presence (C) of the bombesin BB2 receptor antagonist [D-Phe^6, des-Met^{14}]bombesin-(6–14) ethylester (100 nM). Results are expressed as the ratio of emission fluorescence (>510 nm) obtained from cells excited alternately with light of 340 and 380 nm (RU = 340:380). This value is proportional to $[Ca^{2+}]_i$.

followed by 3 × 1 ml washes with ice-cold Tris–HCl buffer. Filters were analysed for radioactivity using a Packard gamma counter. Saturation curves were constructed using concentrations of radioligand from 6.25 to 800 pM, while competition studies were performed using an [^{125}I]bombesin concentration of 100 pM.

All experiments were analysed using GraphPad Prism (ver. 3.02). Saturation and displacement data were fitted to one/two-site binding hyperbola and competition curve-fits, respectively. One- and two-site fits were compared (using an *F*-test) by the program, and data for the plots of statistical best fit are shown. Affinity (K_i) values of competing drugs were calculated using the equation of Cheng and Prusoff (1973).

2.7. Reverse transcription polymerase chain reaction (RT-PCR) assays

Total RNA extracts (Rneasy, Qiagen) from human astrocytes and CHO cells expressing either the human bombesin BB1 receptor or the human bombesin BB2 receptor (to act as positive controls) were analysed by RT-PCR (Superscript II, GIBCO BRL, and Taq DNA polymer-

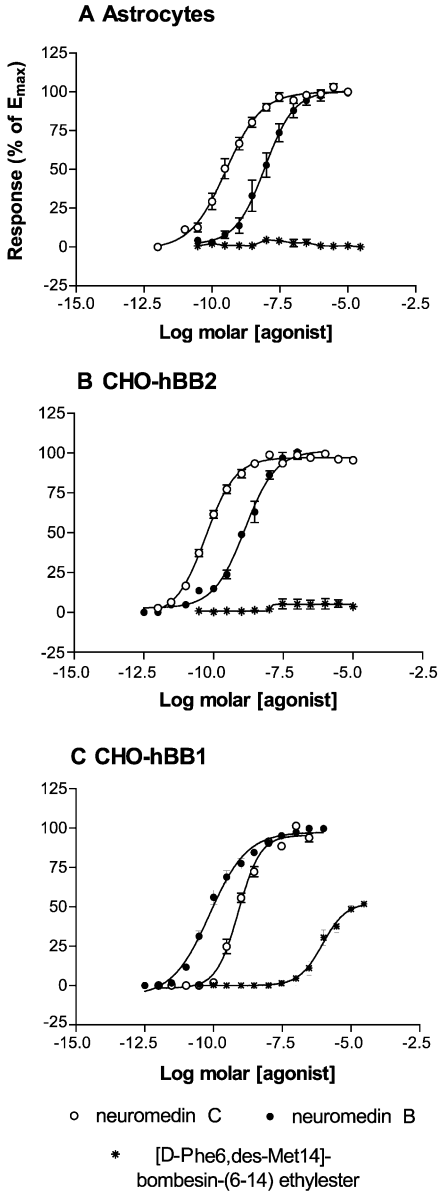

Fig. 4. Mean responses (± S.E.M.) of human astrocytes, hBB1-CHO and hBB2-CHO cells to neuromedin C, neuromedin B, and [D-Phe^6, des-Met^{14}]bombesin-(6–14) ethylester in the FLIPR. Responses represent peak height increases in $[Ca^{2+}]_i$ measured in Fluo-3/4-loaded cells and each curve represents a minimum of three individual determinations.

Table 1
FLIPR analysis of the effects of bombesin receptor peptides

	hBB1-CHO		hBB2-CHO		Astrocyte	
	pEC_{50}	E_{max} (%)	pEC_{50}	E_{max} (%)	pEC_{50}	E_{max} (%)
NMC	9.09 ± 0.04	96 ± 1.6	10.51 ± 0.25	98 ± 1.1	9.77 ± 0.63	100 ± 2.7
NMB	10.59 ± 0.24	99 ± 2.3	8.81 ± 0.12	101 ± 0.8	8.10 ± 0.18	99 ± 3.1
[D-Phe^6, des-Met^{14}]bombesin-(6–14) ethylester	5.85 ± 0.13	55 ± 3.6	–	–	–	–

Efficacy (E_{max}) and potency (pEC_{50}) values are for agonist responses induced by bombesin receptor peptides in the FLIPR. Reponses measured were increases in $[Ca^{2+}]_i$ levels in Fluo-3/4-loaded cells. E_{max} values represent responses as a percentage of the mean curve-fitted E_{max} for neuromedin C. Values shown are means ± S.E.M. taken from at least three individual experiments.

ase, Pharmacia), using pairs of oligoprimers specific for each receptor type.

The primers used were:

BB1: *CTCTGCTGTGGGAGGAAGTC* and ACATTGCCATTTCCTGCTTC;

BB2: *GAAACAGTTCAACACTCAGC* and TGATGAGGCTAAAGGTGGCC;

BB3: *TTGCTCTCTACTGGCTGAGC* and ATCACAGCCAGGGTGGTAAG.

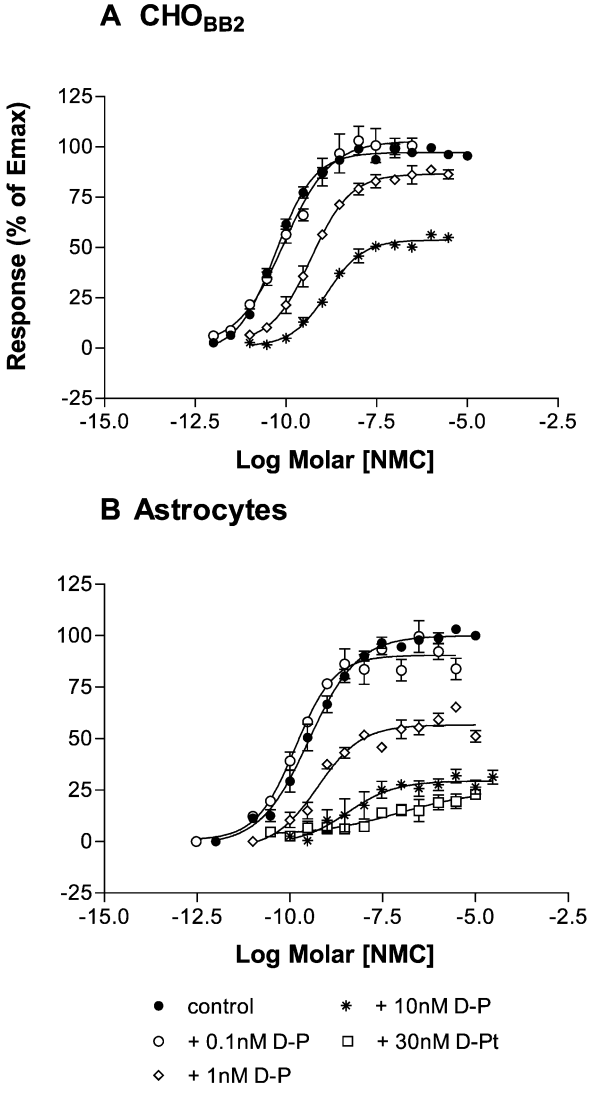

Fig. 5. (A) Inhibition of neuromedin C-induced Ca^{2+} responses in the FLIPR by [D-Phe^6, des-Met^{14}]bombesin-(6–14) ethylester (D-P) in hBB2-CHO cells and (B) human astrocytes. Curves represent mean data (± S.E.M.) obtained from at least three separate experiments.

2.8. Drugs and chemicals

All CHO cell culture media and reagents were purchased from Life Technologies, Paisley, UK. For astrocytes, BioWhitaker products were used. All dye compounds (Fura-2-AM, Fluo-3-AM and Fluo-4-AM) and Pluronic F-127 were obtained from Molecular Probes (OR, USA). Peptide agonists/antagonists were from the following suppliers: neuromedin B, neuromedin C and bombesin, Bachem, (UK); [D-Phe^6, des-Met^{14}]bombesin-(6–14) ethylamide, Penninsula Laboratories (CA, USA); [D-Phe^6, des-Met^{14}]bombesin-(6–14) ethylester, Peptide Products (Salisbury, UK); [^{125}I-Tyr^4]bombesin (2000 Ci/mmol), Amersham Pharmacia Biotech. (UK).

All other chemicals/reagents were obtained from Sigma-Aldrich (Poole, UK).

3. Results

3.1. Confirmation of the identity of human astrocytes

Immunolabeling analysis was undertaken on the cultures derived from primary human astrocytes. These cells all possessed a morphology characteristic of primary astrocytes

Table 2
FLIPR analysis of the effects of [D-Phe^6, des-Met^{14}]bombesin-(6–14) ethylester on neuromedin C responses

	hBB2-CHO		Astrocyte	
	pEC_{50}	E_{max} (%)	pEC_{50}	E_{max} (%)
Neuromedin C alone	10.51 ± 0.25	98 ± 1.1	9.77 ± 0.63	100 ± 2.7
+0.1 nM	10.06 ± 0.02	104 ± 4.5	9.80 ± 0.04	90 ± 4.6
+1 nM	9.36 ± 0.17	87 ± 1.8	9.28 ± 0.17	54 ± 1.5
+10 nM	8.86 ± 0.05	54 ± 0.1	8.44 ± 0.30	28 ± 2.7

Efficacy (E_{max}) and potency (pEC_{50}) values for neuromedin C responses alone or in the presence of [D-Phe^6, des-Met^{14}]bombesin-(6–14) ethylester (+) in the FLIPR. Responses measured represent increases in $[Ca^{2+}]_i$ levels in Fluo-3/4-loaded cells. E_{max} values represent responses as a percentage of the mean curve-fitted E_{max} for neuromedin C. Values shown are means ± S.E.M. taken from 3 to 10 individual experiments.

in culture. Immunolabeling utilising the glial specific marker GFAP revealed strong and specific immunoreactivity in all cultured cells. Examples of two GFAP-stained astrocytes, visualised using a Leica confocal analysis system, are shown in Fig. 1.

3.2. Reverse transcription polymerase chain reaction (RT-PCR) analysis of bombesin receptor mRNA

Preliminary identification of the bombesin receptor subtype present in primary human astrocytes was obtained by PCR studies (Fig. 2). Bands of the correct size were seen for all the receptors using genomic DNA and cDNA extracted from hBB1-CHO and hBB2-CHO cell lines; for cDNA extracted from human astrocytes, the only band observed corresponded to the bombesin BB2 receptor subtype. In the absence of the reverse transcription reaction step, no band was observed. The density of the band suggests the possibility of a substantially lower expression of bombesin BB2 receptor RNA in human astrocytes than in hBB2-CHO cells.

3.3. Single-cell image analysis of human astrocytes

Representative traces obtained from single-cells showing the effects of addition of neuromedin C (a) or neuromedin B (b) at 10 nM are shown in Fig. 3; antagonism of the response to neuromedin C by [D-Phe^6, des-Met^{14}]bombesin-(6–14) ethylester (100 nM) is also shown (Fig. 3C). Analysis of the 340:380 ratio in individual astrocytes revealed that treatment with neuromedin C and neuromedin B (10 nM) increased $[Ca^{2+}]_i$ in 96 ± 2% (n = 8 experiments) and in 78 ± 4% (n = 5 experiments) of cells, respectively. The mean increases of 340:380 ratio units (RU) observed in responding cells were 0.37 ± 0.01 (n = 161) for neuromedin C and 0.26 ± 0.01 (n = 45) for neuromedin B. Removal of extracellular Ca^{2+} did not prevent the response to neuromedin C (0.24 ± 0.01 RU; n = 50) or to neuromedin B (0.29 ± 0.01 RU; n = 53). Cotreatment of cells with neuromedin C (10 nM) and the phospholipase C inhibitor U73122 (1 μM) significantly inhibited the increase in $[Ca^{2+}]_i$ (0.05 ± 0.01 RU; n = 79) compared to cells treated with neuromedin C alone (P < 0.001). Simultaneous treatment of cells with neuromedin C (10 nM) and the antagonist [D-Phe^6, des-Met^{14}]bombesin-(6–14) ethylester (100 nM) substantially reduced the $[Ca^{2+}]_i$ response (0.05 ± 0.02 RU; n = 49) compared to cells treated with neuromedin C alone (P < 0.001). This inhibitory effect of [D-Phe^6, des-Met^{14}]-bombesin-(6–14) ethylester (100 nM) was also observed if cells were stimulated with neuromedin B (data not shown). The removal of [D-Phe^6, des-Met^{14}]bombesin-(6–14) ethylester for a period of 15 min prior to treatment with neuromedin C alone enabled a significant recovery of the Ca^{2+} response to occur (0.23 ± 0.04 RU, n = 49; P < 0.001).

Table 3
Estimated agonist pK_A and antagonist pK_B affinity values

	hBB2-CHO	Astrocyte
pK_A for NMC	8.32 ± 0.14	8.37 ± 0.43
pK_B for [D-Phe^6, des-Met^{14}] bombesin-(6–14) ethylester	9.53 ± 0.24	10.23 ± 0.17

Agonist (pK_A) and antagonist (pK_B) affinity values for neuromedin C and [D-Phe^6, des-Met^{14}]bombesin-(6–14) ethylester, estimated from FLIPR data using a method described by Kenakin (1997). Values shown are means ± S.E.M. taken from three individual curves for each set of data.

Table 4
[^{125}I]Bombesin binding saturation data

	pK_d	B_{max} (pmol/mg protein)
Astrocytes (n = 3)	9.91 ± 0.11 (0.12 nM)	0.240 ± 0.03
hBB2-CHO (n = 3)	10.29 ± 0.21 (0.05 nM)	1.25 ± 0.21
hBB1-CHO (n = 3)	9.43 ± 0.06 (0.37 nM)	1.92 ± 0.19

Saturation data for [^{125}I]bombesin binding to human astrocytes, hBB1-CHO and hBB2-CHO cells. Affinity (pK_d) and density (B_{max}) data are representative of three separate experiments.

3.4. Electrophysiology

In whole-cell current clamp recordings, bath application of 20 nM neuromedin C produced a hyperpolarisation of 32.3 ± 7.2 mV from an initial resting potential of −51.2 ± 3.2 mV that was poorly reversible in nature (n = 8). In whole-cell voltage clamp recordings, 20 nM neuromedin C induced an outward current of 225.3 ± 16.5 pA at −60 mV (n = 7), which had an estimated reversal potential of −82.2 ± 2.1 mV (n = 5). The magnitude of this current was significantly and reversibly reduced by the addition of 2 mM $BaCl_2$ to the bath solution (by 92.3 ± 4.5% (n = 4), P < 0.001).

3.5. FLIPR

3.5.1. Agonist studies

The expression of human recombinant bombesin hBB1 and hBB2 receptors in CHO cells allowed direct comparisons to be made with known bombesin receptor subtypes. Both CHO cells and astrocytes responded to neuromedin B and neuromedin C; however, peak responses in astrocytes were significantly lower than those observed in CHO cells with typical maximum peak height responses corresponding to approximately 10,000 and 35,000 RFU, respectively (data not shown).

Concentration–response curves obtained with bombesin BB1 receptor- and bombesin BB2 receptor-selective agonists demonstrated the expected order of potencies in the transfected CHO cells (Fig. 4 and Table 1). In hBB1-CHO cells, the bombesin BB1 receptor-selective agonist neuromedin B increased $[Ca^{2+}]_i$ with a potency (EC_{50} = 26 pM) over 30-fold higher than that of the bombesin BB2 receptor-specific agonist neuromedin C (EC_{50} = 0.81 nM). Conversely, in hBB2-CHO cells, neuromedin B was 50-fold less potent than neuromedin C (EC_{50} values are 1.54 and 31 pM, respectively). Human astrocytes responded in a pattern

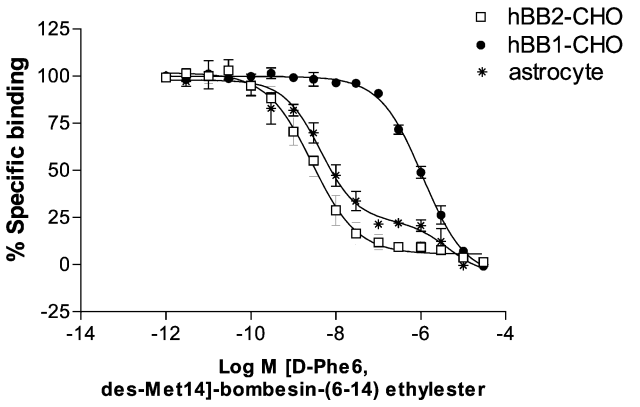

Fig. 6. Inhibition of [^{125}I]bombesin binding to human astrocytes, hBB1-CHO and hBB2-CHO cells by [D-Phe^6, des-Met^{14}]bombesin-(6–14) ethylester. Curves represent the percentage of specific binding in the absence of any competing drug and are generated from three to five individual determinations.

consistent with that seen in hBB2-CHO cells, with neuromedin C 47-fold more potent (EC_{50} = 0.17 nM) than neuromedin B (EC_{50} = 7.94 nM).

To further characterise these responses, the selective bombesin BB2 receptor antagonist [D-Phe^6, des-Met^{14}]bombesin-(6–14) ethylester was used. Although this compound is mainly employed for its antagonist properties at bombesin BB2 receptors, it has been shown to behave as a partial agonist at the BB1 receptor, at higher (μM) concentrations (Ryan et al., 1996). [D-Phe^6, des-Met^{14}]bombesin-(6–14) ethylester was devoid of efficacy in hBB2-CHO cells but produced partial agonist responses of low potency (EC_{50} = 1.41 μM; E_{max} = 55%) in hBB1-CHO cells. Again, consistent with the presence of bombesin BB2 receptors alone, no measurable efficacy was observed with this peptide in human astrocytes.

3.5.2. Antagonist studies

When antagonist experiments were undertaken, it became apparent that [D-Phe^6, des-Met^{14}]bombesin-(6–14) ethylester did not act as a competitive, surmountable antagonist (Fig. 5, Table 2). Incubation with [D-Phe^6, des-Met^{14}]bombesin-(6–14) ethylester not only caused a concentration-dependent rightward shift, but also a suppression of the E_{max} of the neuromedin C response curve. This noncompetitive antagonism was observed in both hBB2-CHO and human astrocytes, but the E_{max} suppression was more marked in astrocytes; here, 10 nM [D-Phe^6, des-Met^{14}]bombesin-(6–14) ethylester reduced the neuromedin C E_{max} to 28% compared to 54% in the hBB2-CHO cell line.

The standard Schild regression methods, as used to measure affinities of competitive antagonists, cannot be applied in such a case, but other methods of analysis can be employed for noncompetitive antagonists where a substantial level of E_{max} suppression ($\geq$ 50%) is encountered (see Methods). For these studies, [D-Phe^6, des-Met^{14}]bombesin-(6–14) ethylester was used at 10 nM for the hBB2-CHO cells, and at 1 nM for astrocytes. The results of these analyses (Table 3) show that [D-Phe^6, des-Met^{14}]bombesin-(6–14) ethylester possesses high affinity in both astrocytes (K_B = 0.06 nM) and hBB2-CHO cells (K_B = 0.3 nM). The affinity estimates for the agonist neuromedin C from these analyses were in the nanomolar range for both astrocytes (K_A = 4.2 nM) and hBB2-CHO cells (4.81 nM).

3.6. [^{125}I]Bombesin binding

[^{125}I]Bombesin is nonselective, binding to both bombesin BB1 and BB2 receptors. The ligand was shown to bind in a saturable manner to both hBB1-CHO and hBB2-CHO cells (see Table 4) possessing a higher affinity at human bombesin BB2 receptor (K_d = 0.05 nM) than the human bombesin BB1 receptor (K_d = 0.37 nM). The K_d value for the binding of [^{125}I]bombesin in astrocytes (K_d = 0.12 nM) was closer to its affinity to the cloned bombesin hBB2 receptor. The density of sites in these cells (B_{max} = 0.24 pmol/mg protein) was considerably lower than in the high expressing recombinant cell lines [1.25 (hBB2-CHO) and 1.92 (hBB1-CHO) pmol/mg protein].

Competition experiments measuring the displacement of a single concentration of [^{125}I]bombesin by [D-Phe^6, des-Met^{14}]bombesin-(6–14) ethylester generated a biphasic curve in astrocytes (Fig. 6). Statistical analysis confirmed this data was best fitted to a two-site model ($P < 0.005$ using F-test). The low Hill coefficient generated from the single site fit (n_H = 0.55) also points to a heterogeneous receptor population. The two-site analysis defined a receptor population consisting of 73% of sites displaced by [D-Phe^6, des-Met^{14}]bombesin-(6–14) ethylester with high affinity (K_i = 2 nM), and 27% displaced with low affinity (K_i = 1.5 μM, see Table 5). Similar analyses on hBB2-CHO cells defined a single population of [^{125}I]bombesin binding sites displaced by [D-Phe^6, des-Met^{14}]bombesin-(6–14) ethylester with high affinity (K_i = 1.3 nM; nH = 0.96). As expected, in

Table 5
Inhibition of [^{125}I]bombesin binding by [D-Phe^6, des-Met^{14}]bombesin-(6–14) ethylester

	pK_i Site 1	Percentage (%)	pK_i Site 2	Percentage (%)	n_H
Astrocytes (n = 3)	8.70 ± 0.08	73 ± 2.5	5.81 ± 0.24	23 ± 2.5	0.55 ± 0.03
hBB2-CHO (n = 5)	8.88 ± 0.18	100	–	–	0.96 ± 0.16
hBB1-CHO (n = 3)	–	–	6.08 ± 0.07	100	0.93 ± 0.05

Inhibition of [^{125}I]bombesin binding to human astrocytes, hBB1-CHO and hBB2-CHO cells by [D-Phe^6, des-Met^{14}]bombesin-(6–14) ethylester. Data have been fitted to single- and two-site models, and where a two-site fit is statistically better, the relevant data are shown. Affinity (pK_i) values, the percent proportion of total binding sites (%) and Hill coefficients (n_H) are shown. Data are representative of three to five separate experiments.

hBB1-CHO cells, [D-Phe^6, des-Met^{14}]bombesin-(6–14) ethylester displaced binding from a homogenous population of sites, with low affinity ($K_i = 0.83$ μM; nH = 0.93). Non-specific [^{125}I]bombesin binding was shown to be less than 10% of the observed total binding in all experiments undertaken for this study.

4. Discussion

The RT-PCR data presented in this study provide strong evidence that bombesin BB2 receptors are expressed in human astrocytes. Significantly, transcript specific only for the bombesin BB2 receptor subtype was detected in human astrocytes. Single-cell analyses have demonstrated $[Ca^{2+}]_i$ increases in response to both neuromedin B and neuromedin C in human astrocytes. This increase in $[Ca^{2+}]_i$ appears to induce a membrane hyperpolarisation in these cells via generation of a Ba^{2+}-sensitive outward current with a reversal close to E_K. Thus, it would appear that in agreement with previous studies using rat astrocytes (Hösli et al., 1993), neuromedin C causes the activation of a K^+ conductance. Similar $[Ca^{2+}]_i$ responses to bombesin have been reported in cultured rat astrocytes (Enkvist et al., 1989), but there has been little progress in defining the intracellular mechanisms or receptor types responsible. In our hands, the rise in $[Ca^{2+}]_i$ was blocked by the phospholipase C inhibitor U73122 and did not require extracellular Ca^{2+}, suggesting a G_q-coupled G-protein-coupled receptor response. Indeed, it is well documented that bombesin BB1 and BB2 receptors couple in this way (Wang et al., 1996; Benya et al., 1992) to release Ca^{2+}.

In experiments utilising single-cell analysis of astrocytes, more prominent responses were observed with neuromedin C compared to neuromedin B, both in terms of magnitude (0.37 vs. 0.26 RU) and frequency (96% vs. 78% of cells). This suggests that these responses are mediated by bombesin BB2 receptors. Further support is provided by the observed antagonist effect of the BB2-receptor antagonist, [D-Phe^6, des-Met^{14}]bombesin-(6–14) ethylester, at a concentration 100-fold lower than that required to reduce significantly [^{125}I]bombesin binding to the hBB1-CHO bombesin receptor. Therefore, although full pharmacological characterisation using single-cell analysis was not practicable, imaging experiments provided functional data to support the RT-PCR data, indicating that the bombesin receptor type present in human astrocytes is bombesin BB2 receptor.

In studies using a 96-well FLIPR assay format, the use of CHO cells expressing the recombinant human bombesin BB1 or bombesin BB2 receptors alongside cultured astrocytes allowed receptor subtype characterisation and comparative studies to be undertaken. As expected, in CHO cells, neuromedin B was substantially more potent than neuromedin C at the bombesin BB1 receptor, with the reverse true at the bombesin BB2 receptor. We observed selectivities for the two agonists (30- to 50-fold) comparable to those from studies measuring functional responses in rat or human tissues (Mantey et al., 1993; Benya et al., 1995; review by Jensen and Coy, 1991).

[D-Phe^6, des-Met^{14}]bombesin-(6–14) ethylester did not exhibit efficacy at bombesin BB2 receptors, but was a partial agonist with low potency (μM) at the bombesin BB1 receptor, exhibiting approximately 50% efficacy, compared to the full agonist neuromedin B. This observation confirms reports of a similar partial agonist effect observed for [D-Phe^6, des-Met^{14}]bombesin-(6–14) ethylester at human bombesin BB1 receptors stably transfected into BALB/3T3 fibroblasts (Ryan et al., 1996). Such partial agonist effects have not been observed for BB1 receptors natively expressed in rat C6 glioma cells (Ryan et al., 1996) or small cell lung carcinoma NCI-H345 cells (Ryan et al., 1993) and are thought to be more apparent in an ''over-expressing'' system. The agonist profile observed in human astrocytes was closely comparable with the hBB2-CHO responses observed and further supports the notion that bombesin receptors in this tissue are of the BB2 type. In both hBB2-CHO cells and astrocytes, [D-Phe^6, des-Met^{14}]-bombesin-(6–14) ethylester produced a concentration-dependent antagonism of the response to neuromedin C that appeared noncompetitive in nature. The greater depression of E_{max} observed in astrocytes is consistent with a reduced receptor reserve due to a lower level of expression of the bombesin BB2 receptor. In addition to the competitive antagonism commonly reported for [D-Phe^6, des-Met^{14}]bombesin-(6–14) ethylester in animal tissue (Maggi et al., 1992, guinea pig and rat urinary bladder; Varga et al., 1991, rat pancreas; Milusheva et al., 1998, cat duodenum), insurmountable antagonism has also been described previously Ryan et al. (1993).

Estimates of the affinity of [D-Phe^6, des-Met^{14}]bombesin-(6–14) ethylester were consistent with published values from analyses of functional bombesin BB2 receptor responses (0.47–9.12 nM, Milusheva et al., 1998; 1.29 nM, Varga et al., 1991). The values derived for affinity of the agonist neuromedin C ($K_A \sim 4$ nM) agree with values for K_i reported for the binding of neuromedin C/gastrin-releasing peptide (1.5, 4.4 and 11.8 nM, Mantey et al., 1993; 6.2 nM, Benya et al., 1995; 1.3 nM, Suman-Chuahan et al., 1995). The high affinity from this study for [^{125}I]bombesin at the hBB2-CHO receptor ($K_d = 50$ pM) agrees with the values reported by other investigators utilising CHO cells (36 pM, Suman-Chuahan et al., 1995) and for bombesin BB2 receptor sites expressed in canine antral gastrin cells (85 pM, Vigna et al., 1990). The lower K_d for this ligand at the hBB1-CHO receptor is also consistent with a value of 287 pM reported by Suman-Chuahan et al. (1995).

Inhibition of [^{125}I]bombesin binding by the selective BB2 receptor antagonist, [D-Phe^6, des-Met^{14}]bombesin-(6–14) ethylester, provided additional data about the bombesin receptor subtypes present in each of the cell types used. The high affinity of this compound against [^{125}I]bombesin

binding in hBB2-CHO cells ($K_i = 1.3$ nM) has been closely matched in numerous other bombesin BB2 receptor studies, with values ranging from 0.5 to 2.1 nM (Benya et al., 1995; Wada et al., 1991; Wu et al., 1995; Staley et al., 1993; Mantey et al., 1993). Similarly, low affinities (0.68–10 μM) have been published for bombesin BB1 receptors (Benya et al., 1995; Wada et al., 1991; Suman-Chuahan et al., 1995), which agree with the value for K_i of 0.83 μM observed in this study. It is possible that the biphasic curves generated for [D-Phe^6, des-Met^{14}]bombesin-(6–14) ethylester in astrocytes could suggest two distinct receptor populations, with the high-affinity population being predominant (73%). However, this is far more likely to be an artefact of the sort commonly associated with peptide agonist radiolabel binding in membranes (Keen, 1999). Indeed, functional studies utilising FLIPR provided no evidence for receptor heterogeneity, and we were unable to detect specific transcript for the human bombesin BB1 or BB3 receptor in astrocytes using RT-PCR.

This study demonstrates that the predominant functional bombesin receptor present on human astrocytes is most closely related to the human bombesin BB2 receptor subtype. The physiological significance of the expression of these receptors on astrocytes is not fully understood, although their presence is highly likely to be integral to the functioning of these cells. Glial cells can sense and respond to many neuroactive substances, and their reactions commonly involve changes in $[Ca^{2+}]_i$ (see Verkhratsky et al., 1998). Increases in glial $[Ca^{2+}]_i$ affect various systems, including K^+ channels (Cooper, 1995; Quandt and MacVicar, 1986), glycogen breakdown (Pentreath et al., 1986), gene expression (Liu and Almazan, 1995; Pende et al., 1994) and neurotransmitter release (O'Connor and Kimelberg, 1993; Parpura et al., 1995). Cross-talk between astrocytes and neurones, whereby an increased $[Ca^{2+}]_i$ in one elicits a response in the other has also been shown to occur (Dani et al., 1992; Dani and Smith, 1995; Porter and McCarthy, 1996; Pasti et al., 1996; Nedergaard, 1994; Parpura et al., 1994). Astrocytes react to injury by altering their biochemical, proliferative and/or structural features, and in doing so play a pivotal role in the defence system of the brain. The mechanisms driving astrocyte responses to injury are poorly understood, but studies have shown that an insult to the brain, such as ischaemia, is associated with rapid increases in astrocytic $[Ca^{2+}]_i$ (Duffy and MacVicar, 1996). It is possible that neuropeptides such as the neuromedins may contribute to these processes. Further efforts into defining the roles of neuropeptide receptors will eventually provide a fuller understanding of how the function of astrocytes is controlled.

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