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Activation of neuromedin B-preferring bombesin receptors on rat glioblastoma C-6 cells increases cellular Ca^{2+} and phosphoinositides

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Recent cloning studies confirm the presence of two subtypes of bombesin (Bn) receptors. In contrast to the gastrin-releasing peptide (GRP)-preferring subtype, which has been widely studied, nothing is known about the cellular mechanisms of the neuromedin B (NMB)-preferring subtype, which occurs widely in the central nervous system and gastrointestinal tissues, partially because of the lack of a cell line with functional receptors. In the present study we have investigated Bn receptors on the rat glioblastoma cell line C-6, reported to contain mRNA of the NMB receptor subtype. Binding of ^{125}I-[D-Tyr^0]NMB to these cells was time- and temperature-dependent, saturable, reversible, and only inhibited by Bn receptor agonists or antagonists. For Bn receptor agonists the relative potencies were: NMB (1.7 nM) $\simeq$ litorin (3 nM) > ranatensin (8 nM) > Bn (19 nM) > neuromedin C (NMC) (210 nM) > GRP (500 nM). These relative affinities were almost identical to those for the NMB receptor subtype on rat oesophageal tissue and for Balb 3T3 cells stably transfected with the NMB receptor subtype. These potencies differed from those for the GRP receptor subtype on rat pancreatic acini [Bn $\simeq$ litorin (4 nM) > ranatensin, NMC, GRP (15–20 nM) $\gg$ NMB (351 nM)]. The relative potencies of four different classes of Bn receptor antagonists were compared. Results from C-6 tumour cells agreed closely with those for binding to the NMB receptor subtype on rat oesophageal tissue and in Balb 3T3 cells stably transfected with this receptor, and differed markedly from those for binding to the GRP receptor subtype on rat pancreatic acini. Four Bn receptor antagonists had a higher affinity for the GRP subtype {[D-Phe^6]Bn-(6–13)ethyl ester (500×), [D-Phe^6][ψ13–14,Cpa^{14}]Bn-(6–14) (70×) (where ψ13–14 refers to the replacement of the –CONH– peptide bond between Leu^{13} and Met^{14} by –CH_2NH_2) [ψ13–14,Leu^{14}]Bn, [D-Phe^6]Bn-(6–13) propylamide (30×)} and two had a higher affinity for the NMB subtype on C-6 cells and transfected cells {[D-Pro^4,D-Trp^{7,9,10}] substance P-(4–11) (9×) and [Tyr^4,D-Phe^{12}]Bn (18×)}. In C-6 tumour cells, Bn receptor agonists caused an increase in cytosolic Ca^{2+} and the generation of inositol phosphates. For both responses, NMB was more than 50-fold more potent than GRP. Neither NMB nor GRP increased cyclic AMP. These results demonstrate that the rat glioblastoma cell line C-6 possesses functional NMB-preferring Bn receptors, and agonist occupation activates phospholipase C, thus increasing cytosolic Ca^{2+} and inositol phosphate formation. Because the interaction of Bn-related peptides with C-6 cell receptors is identical with that reported in other tissues containing the mRNA for the NMB subtype, this cell line should prove useful in exploring further the cellular basis of action of the peptides that interact with this receptor in the central nervous system and various other tissues.

INTRODUCTION

Bombesin (Bn) and structurally related naturally occurring peptides {gastrin-releasing peptide (GRP), neuromedin B (NMB) and neuromedin C [NMC; GRP-(18–27)]} elicit a wide range of biological responses [1–3]. These include release of numerous gastrointestinal hormones [4,5], stimulation of pancreatic enzyme secretion [6], and regulation of central nervous system (CNS) functions such as thermoregulation and maintenance of circadian rhythms [7–9]. The peptides also have the ability to function as a growth factor in 3T3 mouse fibroblasts and small cell lung cancer cells [10–14]. Bn-related peptides have been proposed to have an autocrine mechanism in regulating the growth of small cell lung cancer cells, as both anti-Bn antibodies and bombesin receptor antagonists inhibit growth *in vivo* and *in vitro* [15].

Bn-related peptides can be divided into three structurally related groups, depending on the *C*-terminal sequence [16]. One group comprises Bn and the mammalian peptides GRP and NMC and possesses the *C*-terminal sequence -Gly-His-Leu-Met-NH_2. A second group includes the mammalian peptide NMB, which resembles the frog peptides litorin and ranatensin in possessing a similar *C*-terminus but has a Phe as the penultimate amino acid. The third group has no mammalian equivalent and comprises the frog peptide phyllolitorin, with a serine as the third amino acid from the C-terminus, instead of a histidine as in the other two groups [16].

In previous studies, immunoreactive GRP and NMB and the mRNAs for these peptides have been shown to be widely distributed in the CNS as well as in other mammalian tissues [17–21]. Furthermore, binding and functional studies originally suggested that the two different mammalian Bn-related peptides interact with distinct subtypes of Bn receptors [22–26]. Recently, two distinct subtypes have been cloned [27–29]. One subtype, the GRP-preferring Bn receptor, occurs in the CNS, pituitary cells, pancreatic acinar cells and gastrointestinal smooth muscle cells, and on various cell lines such as murine 3T3 fibroblasts, prostatic

Abbreviations used: Bn, bombesin; GRP, gastrin-releasing peptide; NMB, NMC, neuromedins B and C respectively; CNS, central nervous system; VIP, vasoactive intestinal peptide; DMEM, Dulbecco's modified Eagle's medium; SP, substance P; IBMX, isobutylmethylxanthine; Cpa, *P*-chlorophenylalanine.

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adenocarcinoma cells, small cell lung cancer cells and breast adenocarcinoma cells [23–25,29–36]. The other receptor subtype, the NMB-preferring Bn receptor, has been reported to be the predominant receptor in the CNS and also to occur widely in gastrointestinal smooth muscle [22,25,27]. The cell transduction mechanisms of the GRP-preferring Bn receptor have been extensively studied in 3T3 cells, small cell lung cancer cells and pancreatic acinar cells, where agonists active phospholipase C, generate phosphoinositides, mobilize cellular Ca^{2+}, increase diacylglycerol and activate protein kinase C [11,32,37,38]. In contrast, at present nothing is known about the cellular signalling mechanisms by which agonists for the NMB-preferring Bn receptor alter cell function. This lack of information is due in part to the fact that only quite recently has the existence of this receptor been clearly established [22,23,27]; thus only recently has there been an appreciation of the widespread occurrence, and potential importance of the receptor, especially in the CNS [27,39,40]. Furthermore, the lack of a homogeneous, readily available, preparation of responsive cells that possess this receptor has made it difficult to study the receptor transduction mechanisms. In a recent study [27] in which the NMB-preferring Bn receptor was cloned from rat oesophageal tissue, and various tissues and cell lines were screened for its mRNA, it was found that the rat glioblastoma tumour cell line C-6 expresses the gene for this receptor.

In the present study we have characterized the ability of Bn-related peptides to interact with these cells, compared the results to their interactions with NMB-preferring Bn receptors from oesophageal tissue stably transfected into Balb 3T3 cells, and explored their ability to alter cell function on binding.

MATERIALS AND METHODS

Materials

Male Sprague–Dawley rats (100–200 g) were obtained from Taconic Forms, Germantown, NY, U.S.A. Leupeptin, BSA (Fraction V) and Hepes were from Boehringer Mannheim Biochemicals, Indianapolis, IN, U.S.A.; purified collagenase (type CLSPA; 440 units/mg) was from Worthington Biochemicals, Freehold, NJ, U.S.A.; soybean trypsin inhibitor, chymostatin and bacitracin were from Sigma Chemical Co., St. Louis, MO, U.S.A.; essential vitamin mixture (100× concentrated) was from Microbiological Associates, Bethesda, MD, U.S.A.; and glutamine was from the Media Section, National Institutes of Health, Bethesda, MD, U.S.A. [D-Pro^4,D-$Trp^{7,9,10}$]substance P (SP)-(4–11), NMB, Bn, [Tyr^4]Bn, GRP-(18–27) (NMC), ranatensin, Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2 (CCK-8), vasoactive intestinal peptide (VIP) and SP were from Peninsula Laboratories, Belmont, CA, U.S.A. Synthetic human gastrin-(1–17)-I [desulphated gastrin-(1–17)] was from Cambridge Research Biochemicals, Harston, U.K.; $Na^{125}I$ was from Amersham Co., Arlington Heights, IL, U.S.A.; *myo*-[2-^{3}H]inositol (16–20 Ci/mmol), ^{125}I-succinyl cyclic AMP tyrosine methyl ester and anti-(cyclic AMP) serum (preconjugated to a second antibody) were from New England Nuclear, Boston, MA, U.S.A. Dowex AG 1-X8 anion-exchange resin (100–200 mesh, formate form) was from Bio-Rad, Richmond, CA, U.S.A.; methanol (absolute) and chloroform were from J. T. Baker Chemical, Phillipsburg, NJ, U.S.A., and fura-2 acetoxymethyl ester (fura-2/AM) was from Molecular Probes, Eugene, OR, U.S.A.

Preparation of dispersed rat pancreatic acini

Dispersed acini from rat pancreas were prepared using a modification [41] of the method described previously [42].

Growth of the rat glioblastoma tumour C-6 cell line and transfected Balb 3T3 cells

Stock cultures of rat glioblastoma tumour C-6 cells were obtained from the American Type Culture Collection, Rockville, MD, U.S.A., and were grown in Dulbecco's modified Eagle's medium (DMEM) and 10% fetal bovine serum. Cultures were passaged weekly at subconfluence after trypsin treatment. DMEM and fetal bovine serum were obtained from Gibco, Grand Island, NY, U.S.A. The cells were grown in an atmosphere of 5% CO_2. Balb 3T3 fibroblasts had been stably transfected using calcium phosphate precipitation with a full-length NMB-preferring Bn receptor clone (NMB-R) from rat oesophagus, which was subcloned into a modified version of the pCD2 plasmid [27]. Stably transfected cell lines were selected for resistance to the aminoglycoside G-418 (270 μg/ml) as described previously [27], and were cultured in DMEM supplemented with 10% glutamine and G-418.

Peptides [D-Phe^6]Bn-(6–14) ethyl ester, [ψ13–14,Leu^{14}]Bn, [D-Phe^6]Bn-(6–13) propylamide and [D-Phe^6,ψ13–14,Leu^{13},Cpa^{14}]Bn-(6–14) were synthesized using solid phase methods as described previously [43,44]. ψ13–14 refers to the replacement of the –CONH– peptide bond between Leu^{13} and Met^{14} by $-CH_2NH_2-$. Briefly, introduction of the reduced peptide bonds was carried out on methylbenzhydrylamine resin (Advanced Chem Tech, Louisville, KY, U.S.A.) by standard methods [45]. The alkylamide analogue was synthesized in a standard Leu-*O*-polystyrene resin using tosyl group protection for the imidazole group of His. [D-Tyr^0]NMB was prepared by standard solid phase methods. Peptides were purified on a column (2.5 cm × 90 cm) of Sephadex G-25, followed by elution with linear gradients of acetonitrile in 0.1% trifluoroacetic acid using an Eldex Chromatrol gradient controller (flow rate approx. 1 ml/min) and columns (1.5 cm × 50 cm) of Vydac C_{18} silica (10–15 mM). Peptides were further purified by re-chromatography on the same column, with slightly modifications to the gradient conditions when necessary to obtain greater than 97% purity.

Preparation of ^{125}I-[Tyr^4]Bn and ^{125}I-[D-Tyr^0]NMB

^{125}I-[Tyr^4]Bn (2000 Ci/mmol) was prepared using Iodogen and purified by h.p.l.c. using the modification [22,23] of the method described previously [32]. ^{125}I-[D-Tyr^0]NMB was prepared by adding 0.4 μg of Iodogen to 8.0 μg of [D-Tyr^0]NMB with 2 mCi of $Na^{125}I$ in 20 μl of 0.5 M-potassium phosphate buffer (pH 7.4). After incubation at 22 °C for 6 min, 300 μl of 1.5 M-dithiothreitol was added and the reaction mixture was incubated at 80 °C for 60 min. Free ^{125}I was separated by applying the reaction mixture to a Sep-Pak (Waters Associates, Milford, MA, U.S.A.), which was prepared by washing with 5 ml of methanol, 5 ml of 0.1% trifluoroacetic acid and 5 ml of water. Free ^{125}I was eluted by 5 ml of 0.1% trifluoroacetic acid, and the radiolabelled peptide by 200 μl sequential elutions (× 10) with 60% acetonitrile in 0.1% trifluoroacetic acid. Radiolabelled peptide was separated from unlabelled peptide by combining the three elutions (0.6 ml) containing the highest radioactivity and applying them to reverse-phase h.p.l.c. (Waters Associates; model 204 with a Rheodyne injector) with a μBondaPak column (0.46 cm × 25 cm). The column was eluted with a linear gradient of acetonitrile in 0.1% (v/v) trifluoroacetic acid, from 24 to 72% (v/v) acetonitrile in 60 min with a flow rate of 1.0 ml/min. ^{125}I-[D-Tyr^0]NMB was stored with 1% (w/v) BSA at −20 °C and was stable for at least 6 weeks.

Binding of ^{125}I-[D-Tyr^0]NMB to rat glial C-6 cells and NMB-R-transfected cells

C-6 cells or Balb 3T3 cells transfected with NMB-R were

suspended in standard incubation buffer containing 50 mM-Hepes, 130 mM-NaCl, 7.7 mM-KCl, 5 mM-$MgCl_2$, 1.0 mM-EGTA, 4 μg of leuceptin/ml, 2 μg of chymostatin/ml, 0.1% (w/v) bacitracin, 0.02% (w/v) trypsin inhibitor, 0.02% (w/v) benzamidine hydrochloride and 0.2% (w/v) BSA. Incubations contained 75 pM-^{125}I-[D-Tyr^0]NMB for C-6 cells (15×10^6 cells/ml) or 50 pM-^{125}I-[D-Tyr^0]NMB for NMB-R-transfected cells (5×10^6 cells/ml) and were for 60 min at 22 °C. Non-saturable binding for ^{125}I-[D-Tyr^0]NMB was the amount of radioactivity associated with C-6 cells or NMB-R-transfected cells when the incubation mixture contained 1 μM-NMB. Non-saturable binding was < 15% of total binding in all experiments. All values in this paper are for saturable binding, i.e. total binding minus non-saturable binding. To determine the affinity of ^{125}I-[D-Tyr^0]NMB for the NMBR receptor on C-6 cells, these cells (15×10^6 cells/ml) were incubated with various concentrations of ^{125}I-[D-Tyr^0]NMB (3 pM to 5 nM) alone or with 1 μM-NMB. Saturable binding was analysed using a non-linear least-squares curve-fitting program (LIGAND) [46]. K_d was calculated using the second-order association rate constant (K_1) and the rate constant for dissociation (K_{-1}) as K_{-1}/K_1. The association rate constant was calculated by pseudo-first order kinetics using the equation $\ln[B_{eqm.}/(B_{eqm.}-B)] = K_{ob.}t$, where B is the amount bound at time t, $K_{ob.}$ is the observed initial rate constant and $B_{eqm.}$ is the amount specifically bound at equilibrium. The second-order rate constant (K_1) was determined from the equation $K_1 = (K_{ob.}-K_{-1})/[^{125}$I-[D-Tyr^0]NMB], where the concentration of ^{125}I-[D-Tyr^0] was 0.05 nM.

Binding of ^{125}I-[Tyr^4]Bn to the GRP receptor on rat pancreatic acini

Binding was performed as described previously [32,47]. Briefly, incubations contained 50 pM-^{125}I-[Tyr^4]Bn and were for 60 min at 37 °C. Non-saturable binding of ^{125}I-[Tyr^4]Bn was the amount of radioactivity associated with the acini in the presence of 50 pM-^{125}I-[Tyr^4]Bn and 1 μM-Bn. Non-saturable binding was < 10% of total binding in all experiments.

Measurement of changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)

Rat glioblastoma C-6 cells (5×10^6 cells/ml) were suspended in standard incubation solution (without essential vitamin mixture) and incubated with 2 μM-fura-2/AM for 45 min at 37 °C. Fura-2-loaded cells were then washed three times with 45 ml of incubation solution and resuspended in standard incubation solution. For measurement of $[Ca^{2+}]_i$, 2.0 ml samples were removed at selected time points for fluorescence readings. The fluorescence of fura-2-loaded glioblastoma cells was measured using a Delta PTI Scan 1 Spectrofluorimeter (PTI Instruments, Gaithersburg, MD, U.S.A.) that had provision for maintaining the incubation temperature at 37 °C and for mixing the cell suspension with a magnetic stirrer in the fluorimeter cuvette. Fluorescence was measured at 500 nm after excitation at 340 nm (F_{340}) and 380 nm (F_{380}). Autofluorescence of the unloaded cells was subtracted. $[Ca^{2+}]_i$ was calculated by the method of Grynkiewicz *et al.* [48] using the formula: $[Ca^{2+}] = K_d \times (R-R_{min.})/(R_{max.}-R) \times S_f/S_b$, where K_d is the affinity of fura-2 for Ca^{2+} (determined to be 225 nM), R is F_{340}/F_{380}, i.e. the ratio of the fluorescence with the two excitation wavelengths, $R_{max.}$ is the F_{340}/F_{380} ratio in a saturated Ca^{2+} environment after addition of 0.1% Triton, $R_{min.}$ is the fluorescence ratio at virtually zero calcium (addition of 25 mM-EGTA), S_f is the F_{380} at zero $[Ca^{2+}]_i$ and S_b is the F_{380} at saturated $[Ca^{2+}]_i$.

Measurement of changes in phosphoinositides in C-6 cells

Phosphoinositides in rat glioblastoma C-6 cells were radiolabelled as described previously [49] by incubating cells (1×10^7 cells/ml) with 100 μCi of *myo*-[2-^{3}H]inositol/ml at 37 °C for 2 h. Cells were washed and incubated with 10 mM-LiCl for 15 min and then for 30 min with various concentrations of agonists.

The procedures for the separation of water-soluble [^{3}H]inositol phosphates were performed as described previously [50], with the following modifications. The columns were first washed with 4 ml of distilled water to remove [^{3}H]inositol, then with 1.25 ml of 5 mM-disodium tetraborate and 60 mM-sodium formate to remove [^{3}H]glycerophosphoinositol, and finally with 4 ml of 100 mM-formic acid and 1.0 M-ammonium formate to remove the total [^{3}H]inositol phosphates. The eluates were then assayed for radioactivity after the addition of Ready-Gel scintillation solution to each vial.

Measurement of changes in cyclic AMP in C-6 cells

Cyclic AMP was determined by radioimmunoassay using the procedure of Harper & Brooker [51], as described previously [52]. Incubations contained 0.5 ml of cell suspension (20×10^6/ml) and were at 37 °C for 30 min.

RESULTS

To characterize the optimal conditions for binding of ^{125}I-[D-Tyr^0]NMB to rat glioblastoma C-6 tumour cells, the time- and temperature-dependence of binding were determined (Fig. 1). Maximal binding occurred at 22 °C, with half-maximal and maximal binding occurring at 5 min and 30 min respectively. Adding 1 μM-NMB caused an 87% decrease in maximal binding (Fig. 1). At 4 °C binding was relatively slower, reaching only 70% of the amount seen at 22 °C by 60 min. At 37 °C binding was rapid, reaching a peak by 10 min (which was however only 60% of the maximal binding at 22 °C) and then decreasing at longer incubation times (Fig. 1). In separate experiments the observed association rate constant, $K_{ob.}$, was determined

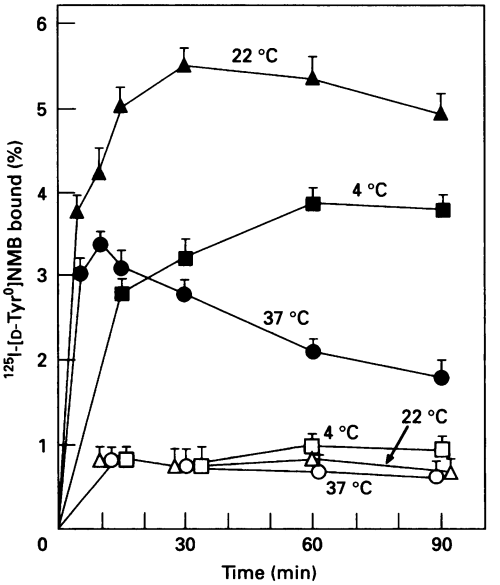

Fig. 1. Time- and temperature-dependent binding of ^{125}I-[D-Tyr^0]NMB to rat glioblastoma C-6 tumour cells

Rat glioblastoma C-6 tumour cells ($15–10^6$/ml) were incubated with 75 pM-^{125}I-[D-Tyr^0]NMB alone (■, ▲, ●) or plus 1 μM-NMB (□, △, ○) at the indicated temperatures and for the indicated times. Results are expressed as percentages of the added radioactivity bound. In each experiment, each value was determined in duplicate and results given are means ± S.E.M. of three separate experiments.

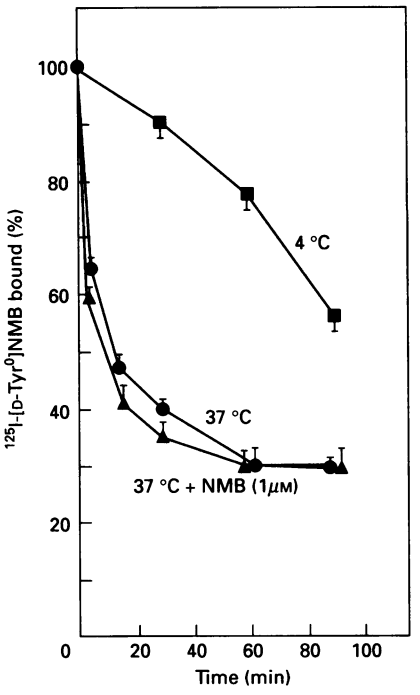

Fig. 2. Dissociation of bound ^{125}I-[D-Tyr^0]NMB from rat glioblastoma C-6 tumour cells

Rat glioblastoma C-6 cells (20×10^6 cells/ml) were incubated at 22 °C with 75 pM-^{125}I-[D-Tyr^0]NMB for 90 min. At that time aliquots were diluted 50-fold with standard incubation buffer at 4 °C or 37 °C and incubated at the indicated temperatures for the indicated times. The results are expressed as percentages of saturable binding at the beginning of the second incubation. In each experiment, each value was determined in duplicate and each point is the mean ± S.E.M. of three separate experiments.

Table 1. Comparison of the ability of various peptides to inhibit binding of ^{125}I-[Tyr^4]Bn to rat pancreatic acinar cells and binding of ^{125}I-[D-Tyr^0]NMB to Balb cells transfected with NMB receptors from rat oesophageal muscle and rat glioblastoma C-6 tumour cells

Balb 3T3 cells were stably transfected with NMB-preferring Bn receptors (NMB-R) from rat oesophagus as described in the Materials and methods section. K_i values for binding of the various peptides were calculated by the method of Cheng & Prusoff [59]. Each cell was incubated with either ^{125}I-[Tyr^4]Bn or ^{125}I-[D-Tyr^0]NMB alone or along with one of the listed peptides. Each value is the mean ± S.E.M. of a minimum of four separate experiments. ψ13–14 refers to the replacement of the –CONH– peptide bond between Leu^{13} and Met^{14} by $-CH_2NH_2-$ as described previously [43,45]; Cpa, *p*-chlorophenylalanine.

	K_i (nM)		
Peptide	Rat pancreas	NMB-R-transfected Balb 3T3 cells	C-6 cells
GRP	15±3	440±70	500±27
NMC	20±12	140±13	210±17
NMB	351±28	3.6±0.6	1.7±0.1
Litorin	5.7±1.3	6±1	3±0.3
Bombesin	4±1	34±2	19±2.5
Ranatensin	14±7	13±1.6	8±0.4
[D-Pro^4,D-Trp^{7,9,10}]-SP-(4–11)	13000±2000	2510±610	1410±90
[Tyr^4,D-Phe^{12}]Bn	54700±11300	1870±130	3330±240
[ψ13–14,Leu^{14}]Bn	434±65	32500±6000	13160±780
[D-Phe^6,ψ13–14,Cpa^{14},-Cpa^{14}]Bn-(6–14)	41±4.8	2710±240	2540±940
[D-Phe^6]Bn(6–13) propylamide	21±2	400±340	650±140
[D-Phe^6]Bn-(6–13) ethyl ester	5.3±31.1	1180±300	2530±150

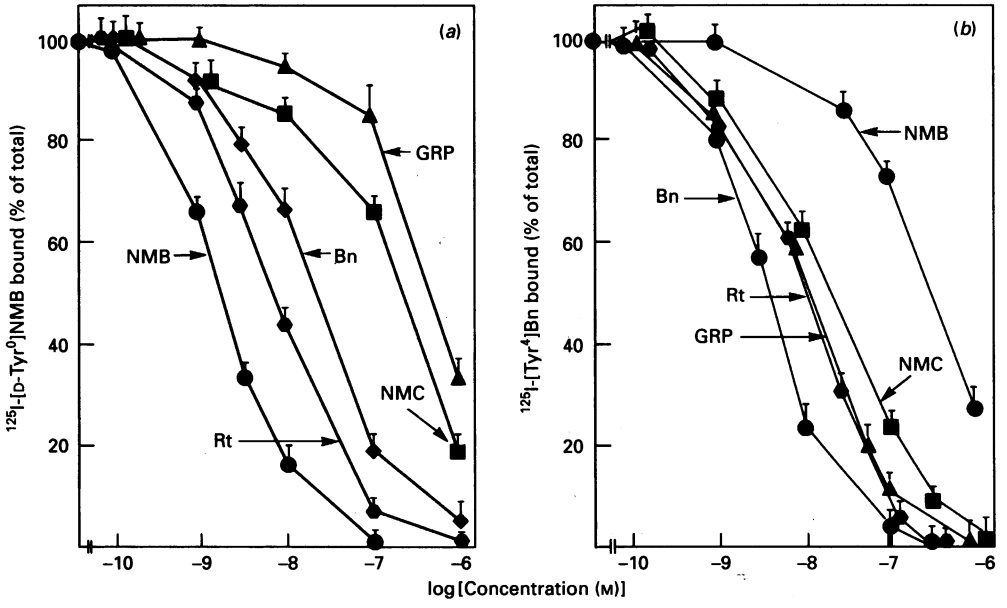

Fig. 3. Ability of various naturally occurring Bn-related receptor agonists to inhibit binding of ^{125}I-[D-Tyr^0]NMB to rat glioblastoma C-6 tumour cells (*a*), or binding of ^{125}I-[Tyr^4]Bn to rat pancreatic acini (*b*)

The C-6 cells were incubated with 75 pM-^{125}I-[D-Tyr^0]NMB and rat pancreatic acini with 50 pM-^{125}I-[Tyr^4]Bn alone or with the indicated concentrations of the Bn-related peptide agonists. Data are expressed as percentages of the saturably bound radioactivity in absence of non-radioactive peptide. In each experiment each value was determined in duplicate and results given are means ± S.E.M. from four separate experiments. Abbreviation: Rt, ranatensin.

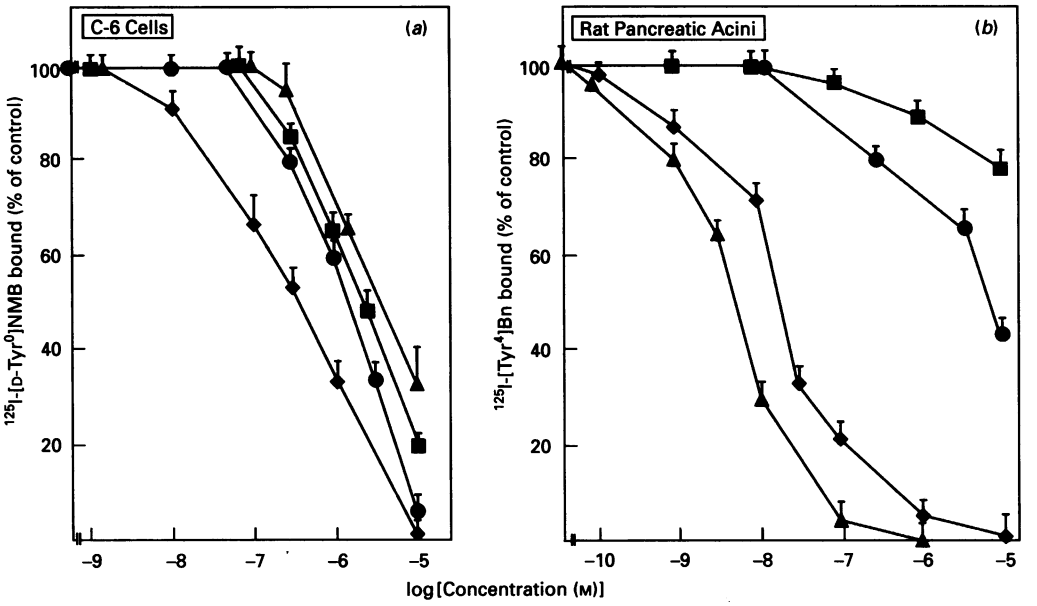

Fig. 4. Ability of various Bn receptor antagonists to inhibit binding of ^{125}I-[D-Tyr^0]NMB to rat glioblastoma tumour cells (*a*), or binding of ^{125}I-[Tyr^4]Bn to rat pancreatic acini (*b*)

The C-6 tumour cells were incubated with 75 pM-^{125}I-[D-Tyr^0]NMB and rat pancreatic acini with 50 pM-^{125}I-[Tyr^4]Bn, alone or with the indicated concentrations of Bn receptor antagonists: ◆, [D-Phe^6]Bn-(6–13) propylamide; ●, [D-Pro^4,D-Trp^{7,9,10}]SP-(4–11); ■, [Tyr^4,D-Phe^{12}]Bn; ▲, [D-Phe]Bn-(6–13) ethyl ester. Data are expressed as percentages of saturably bound radioactivity in the absence of non-radioactive peptide. In each experiment, each value was determined in duplicate and results given are means ± S.E.M. from four separate experiments.

by measuring saturable binding at 2.5, 5, 7.5, 10, 15, 20, 25 and 30 min. The observed initial rate constant was 0.099 ± 0.006 min^{-1}.

To investigate the kinetics of binding further, the reversibility of binding of ^{125}I-[D-Tyr^0]NMB was examined (Fig. 2). Dissociation of bound ^{125}I-[D-Tyr^0]NMB at 37 °C was rapid, with half-maximal dissociation at 15 min and 70% dissociation at 60 min. Adding 1 μM-NMB did not change the dissociation at 37 °C. The rate constant of dissociation (K_{-1}) for the initial dissociation slope was 0.088 ± 0.007 min^{-1}. Lowering the temperature to 4 °C slowed the dissociation of ^{125}I-[Tyr^0]NMB, such that only 23% had dissociated by 60 min (Fig. 2). To examine the specificity of binding of ^{125}I-[D-Tyr^0]NMB to rat glioblastoma C-6 cells, various agents were tested for their ability to inhibit binding. Binding of ^{125}I-[D-Tyr^0]NMB was decreased 96% by 0.1 μM-NMB and 70% by 0.1 μM-Bn or 1 μM-GRP, but was not inhibited by the structurally unrelated peptides CCK-8, gastrin-17-I, VIP or SP (all at 0.1 μM) (results not shown).

To characterize further the Bn receptors identified on the rat glioblastoma C-6 tumour cells, we tested the ability of various Bn-related peptide agonists, as well as Bn receptor antagonists, to inhibit binding of ^{125}I-[D-Tyr^0]NMB (Fig. 3). NMB was most potent in inhibiting binding of ^{125}I-[D-Tyr^0]NMB, causing detectable inhibition at 0.1 nM, half-maximal inhibition at 1.7 nM and complete inhibition at 0.1 mM (Fig. 3*a*; Table 1). Ranatensin was 3-fold less potent than NMB, and NMB was 10-fold, 130-fold and 300-fold more potent than Bn, NMC and GRP respectively (Fig. 3*a*; Table 1). Computer analysis of the ability of NMB to inhibit binding of ^{125}I-[D-Tyr^0]NMB using a least-squares curve-fitting program (LIGAND) [46] demonstrated that the data best fitted a single-binding-site model, with a K_d of 1.7 ± 0.2 nM for NMB and a $B_{max.}$ of 30 ± 1 fmol/mg of protein or 2117 ± 304 receptors/cell. These results agree closely with those calculated from saturation studies using ^{125}I-[D-Tyr^0]NMB at 3 pM–5 nM, in which a single-binding-site model gave the best fit with a K_d of 1.6 ± 0.6 nM (results not shown). Both of these K_d values are also in relatively close agreement with the kinetic K_d value of 0.94 ± 0.13 nM.

These results were compared with the ability of naturally occurring Bn receptor agonists to interact with Balb 3T3 cells which had been transfected using the oesophageal NMB-preferring bombesin receptor, or with the GRP-preferring bombesin receptors which have been described on rat pancreatic acini [6,22]. The abilities of NMB and structurally related agonists to inhibit binding of ^{125}I-[D-Tyr^0]NMB to oesophageal NMB receptors in transfected 3T3 cells showed very close agreement with the results of binding to rat glioblastoma C-6 cells (Table 1). Specifically, in each case the relative abilities of the peptides to inhibit binding of ^{125}I-[D-Tyr^0]NMB were NMB = litorin > ranatensin > bombesin ≫ NMC > GRP, and there was very close agreement in the affinities of each agonist for the two receptors (Table 1). In contrast, the affinities of the agonists for the GRP-preferring bombesin receptors on rat pancreatic acini differed markedly from those for receptors on the C-6 cells (Fig. 3). As described previously [22], with rat pancreatic acini the relative potencies were bombesin = litorin > ranatensin = GRP = NMC ≫ NMB. Thus the NMB-preferring Bn receptors on rat glioblastoma C-6 tumour cells had, in comparison with those on rat pancreatic acini, a 205-fold greater affinity for NMB, an approximately equal affinity for litorin and ranatensin, and 4-fold, 10-fold and 34-fold lower affinities for bombesin, NMC and GRP respectively (Table 1).

A number of different classes of Bn receptor antagonists have been described [53–55], some of which have been reported to distinguish between Bn receptor subtypes [23,55]. Therefore, to further characterize the receptors for Bn-related peptides on C-6 cells, the ability of a potent member of each of the four different classes of Bn receptor antagonists [55] to interact with Bn receptors on these cells was explored, and compared with their interactions on NMB-R-transfected cells and rat pancreatic acini with GRP-preferring Bn receptors. Each of the antagonists inhibited binding of ^{125}I-[D-Tyr^0]NMB to C-6 cells, although with

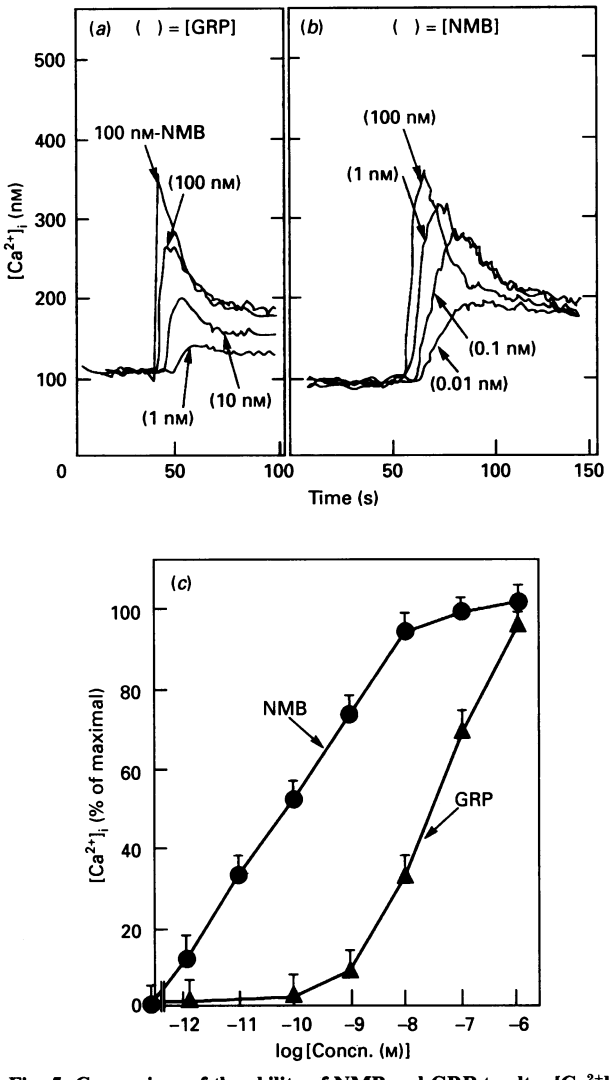

Fig. 5. Comparison of the ability of NMB and GRP to alter $[Ca^{2+}]_i$ in rat glioblastoma C-6 tumour cells

C-6 tumour cells were loaded with 2 μM-fura-2/AM, washed and resuspended in standard incubation buffer as described in the Materials and the methods section. (*a*, *b*) Time courses of the effect of various concentrations of GRP or NMB on $[Ca^{2+}]_i$. Results of this experiment are representative of five others. (*c*) Increases in $[Ca^{2+}]_i$ caused by various concentrations of GRP or NMB are expressed as percentages of that caused by 1 μM-NMB, a maximally effective concentration. Results are means ± S.E.M. of five experiments. In these experiments the basal $[Ca^{2+}]_i$ was 100 ± 10 nM, and the 1 μM-NMB-stimulated value was 367 ± 36 nM.

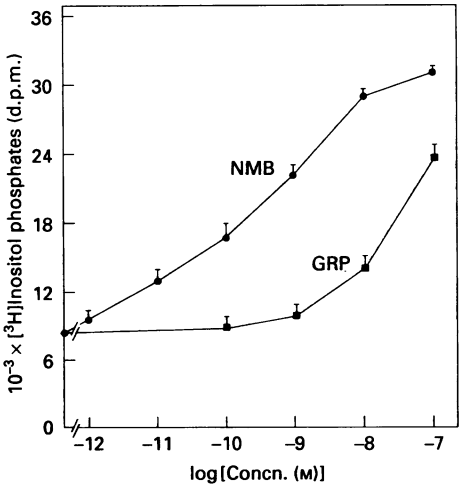

Fig. 6. Ability of NMB and GRP to stimulate changes in [^{3}H]inositol phosphates in rat glioblastoma C-6 cells

C-6 cells (10×10^6/ml) were incubated with *myo*-[2-H^3]inositol for 2 h, washed and incubated either alone or with various concentrations of NMB or GRP for 30 min at 37 °C. [^{3}H]Inositol phosphates were determined as described in the Materials and methods section. Results are from three experiments, and are means ± S.E.M.

relatively low affinity (Fig. 4*a*; Table 1). [D-Phe^6]Bn-(6–13) propylamide (K_i 650 nM; Fig. 4; Table 1) was the most effective and was 2-fold more potent than [D-Pro^4,D-Trp^{7,9,10}]SP-(4–11), 4-fold more potent than [D-Phe^6,ψ13–14,Cpa^{11}]Bn-(6–14) and [D-Phe^6]Bn-(6–13) ethyl ester, 5-fold more potent than [Tyr^4, D-Phe^{12}]Bn, and 20-fold more potent than [ψ13–14,Leu^{14}]Bn. These results were in close agreement with the relative abilities of these antagonists to interact with NMB-R-transfected Balb 3T3 cells (Table 1). In contrast to the results in C-6 cells and NMB-R-transfected cells, as reported previously [24,45,53–55] a number of these antagonists had high affinity for GRP-preferring Bn receptors on rat pancreatic acini. [D-Phe^6]Bn-(6–13) ethyl ester (K_i, 5.3 nM) had the highest affinity, and was 4-fold more potent than [D-Phe^6]Bn-(6–13) propylamide, 8-fold more potent than [D-Phe^6,ψ13–14, Cpa^{14}]Bn-(6–14), 80-fold more potent than [D-Pro^4,D-Trp^{7,9,10}]SP-(4–11), and 10000-fold more potent than [Tyr^4,D-Phe^{12}]Bn (Fig. 4*b*; Table 1). Therefore [D-Phe^6]Bn-(6–13) ethyl ester was 500-fold more selective for GRP-preferring Bn receptors on rat pancreatic acini than for NMB-preferring receptors on rat glioblastoma or C-6 tumour cells. [D-Phe^6,ψ13–14,Cpa^{14}]Bn-(6–14) was 60-fold more selective and [D-Phe^6]Bn-(6–13) propylamide and [ψ13–14,Leu^{14}]Bn were 30-fold more selective for GRP-preferring Bn receptors on rat pancreatic acini than for NMB-preferring Bn receptors on C-6 cells or NMB-R-transfected cells (Table 1).

In previous studies of GRP-preferring Bn receptors in a number of different tissues, receptor occupation by agonists activated phospholipase C, resulting in the breakdown of phosphoinositides and the mobilization of cellular Ca^{2+} [11,32,37,38]. To investigate whether agonist occupation of NMB-preferring Bn receptors on rat glioblastoma tumour C-6 cells resulted in similar changes in cellular mediators, the ability of NMB and GRP to alter $[Ca^{2+}]_i$ (Fig. 5) and increase [^{3}H]-inositol phosphates (Fig. 6) was investigated. A detectable increase in $[Ca^{2+}]_i$ was seen with 1 pM-NMB and 1 nM-GRP (Fig. 5). Maximally effective concentrations of both peptides caused a 3.5-fold increase in $[Ca^{2+}]_i$, with NMB and GRP causing half-maximal increases at 0.1 nM and 30 nM respectively and maximal increases at 10 nM and 1000 nM respectively (Fig. 5). NMB and GRP both also increased the generation of [^{3}H]inositol phosphates, with NMB causing a detectable effect at 1 pM, a half-maximal effect at 0.38 nM and maximal 4-fold increase at 10 nM (Fig. 6). GRP caused a detectable increase at 1 nM and an effect equal to the half-maximal effect of NMB at 40 nM. Therefore, for stimulating an increase both in $[Ca^{2+}]_i$ and in the generation of [^{3}H]inositol phosphates, NMB was > 50-fold more potent than GRP. Neither NMB nor GRP increased cyclic AMP in C-6 tumour cells in the presence of the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX). Specifically, with rat glioblastoma cells (20×10^6 cells/ml) in the presence of 0.1 mM-IBMX with no additions, NMB (1000 nM) or GRP (1000 nM), the cellular cyclic AMP values were 33 ± 6, 25 ± 2 and 25 ± 3 pmol/mg of protein respectively (mean ± S.E.M., $n = 3$), which were not significantly different from each other.

DISCUSSION

The present study demonstrates that the rat glioblastoma tumour cell line C-6 possesses specific high-affinity functional Bn receptors that are of the NMB-preferring subtype and that closely resemble those described previously in oesophageal muscularis mucosa [22,23,27]. The evidence that the binding of radiolabelled ^{125}I-[D-Tyr^0]NMB was to specific receptors was that it was time- and temperature-dependent, saturable, reversible, and only inhibited by Bn receptor agonists or antagonists and not by other peptides such as CCK, gastrin, SP or VIP that do not interact with Bn receptors [32,56]. A number of results demonstrate that the Bn receptors on C-6 tumour cells are the NMB-preferring subtype, and are clearly different from the GRP-preferring subtype that has been extensively characterized in the CNS, and on pituitary cells, pancreatic acinar cells and various cell lines such as 3T3 cells, small cell lung cancer cells, prostatic and breast cancer cells [27–36].

First, the C-6 tumour cells bound radiolabelled NMB, whereas in previous studies GRP-preferring Bn receptors such as those on rat pancreas will not bind this ligand because of their lower affinity for NMB (i.e. 300 nM) [22].

Secondly, the dose–inhibition curves for the various naturally occurring Bn receptor agonists demonstrated a relative order of potency of NMB $\simeq$ litorin > ranatensin > bombesin > NMC $\gg$ GRP. This relative order of agonist potency is similar to that described for NMB-preferring Bn receptors originally described in rat oesophageal tissue [22], and is similar to that exhibited by these agonists in the present study and a previous study [27] for NMB-preferring Bn receptors from rat oesophageal tissue that had been stably transfected into Balb 3T3 cells. The absolute agonist potencies are very similar in C-6 cells and the transfected cells (Table 1), and are in general 10-fold lower than those originally described in rat oesophageal mucosa [22]. Because the C-6 cells and oesophageal muscularis mucosa have the same NMB receptor [27], yet differ in absolute affinity, this suggests that absolute differences in affinity may be due to the different methodologies used to measure the binding constants with oesophageal muscularis mucosa. Tissue sections mounted on glass slides were used, whereas with both C-6 cells and transfected cells a centrifugation method after incubation of dispersed cells in solution was used. The relative order of potency of agonists differed markedly from that of the agonists in interacting with GRP-preferring Bn receptors from rat pancreas or 3T3 cells; in the latter case the relative order of potency was bombesin $\simeq$ ranatensin, litorin, GRP, NMC $\gg$ NMB.

Thirdly, four different classes of Bn receptor antagonists have been described, and various members of these different classes of antagonists have markedly different affinities for the two subtypes of Bn receptors [23,53–55]. In the present study the relative affinities of various members of these four classes of antagonists for Bn receptors on C-6 tumour cells agreed closely with those for receptors on rat oesophageal tissue stably transfected into Balb 3T3 cells, as well as those reported previously for NMB-preferring Bn receptors on rat oesophageal muscularis mucosa [22,27,55], but differed significantly from those for the GRP-preferring Bn receptors [23,55]. Specifically, in previous studies the D-amino acid-substituted SP or SP-4-11 analogues, and the D-Phe^{12}-substituted Bn analogues that function as Bn receptor antagonists at both Bn receptor subtypes, were found to have higher affinities for NMB- than for GRP-preferring Bn receptors [23,55]. A similar result was found in the present study in the C-6 tumour cells. Similar to the NMB-R-transfected Balb 3T3 cells, [D-Pro^4,D-Trp^{7,9,10},Leu^{11}]SP-(4–11) had a 9-fold higher affinity, and [Tyr^4,D-Phe^{12}]Bn a 20-fold higher affinity, for NMB-preferring than for GRP-preferring Bn receptors on rat pancreas. In contrast, various pseudopeptide analogues of Bn, as well as des-Met^{14}-Bn analogues that function as Bn receptor antagonists for both subtypes, are reported to have a much higher affinity for GRP- than for NMB-preferring receptor subtypes [23,55]. In the present study, the affinities of four analogues from these two antagonist classes agreed very closely for C-6 tumour cells and NMB-R-transfected Balb 3T3 cells, and for each analogue the affinity was > 20-fold lower than that for GRP-preferring Bn receptors on rat pancreatic acini. The des-Met^{14}-Bn analogue [D-Phe^6]Bn-(6–13) ethyl ester appears to be especially discriminating, in that it is reported to have an affinity almost equal to those of Bn and GRP themselves for GRP-preferring receptors, and a much lower affinity for NMB-preferring receptors [23,55]. Similarly, in the present study this analogue was found to have a 500-fold higher affinity for the GRP-preferring Bn receptor on rat pancreas than for the receptors on C-6 tumour cells.

Even though numerous studies, including recent ones using a specific cDNA probe for NMB-preferring Bn receptors, suggest that these receptors are widely distributed in the CNS and in gastrointestinal tissues [22–27,29], there are no studies on the cellular basis of action of agonists at this receptor. Previous extensive studies demonstrating that Bn-related peptides alter cell function by altering ion fluxes, activate phospholipase C with the generation of inositol phosphates and mobilization of cellular Ca^{2+}, and activate protein kinase C, were all performed in 3T3 cells, rat pancreatic acinar cells or small cell lung cancer cells, all of which possess GRP-preferring Bn receptors [11,32,37,38]. The present study demonstrates that occupation of the NMB-preferring receptor by agonists activates phospholipase C, causing the generation of phosphoinositides and mobilization of cellular Ca^{2+}. One study [57] has demonstrated that, in the presence of a phosphodiesterase inhibitor or in the presence of forskolin, Bn-related peptides can increase cyclic AMP in 3T3 cells which possess GRP-preferring receptors. In the present study in the presence of 0.1 mM-IBMX, neither GRP nor NMB altered cyclic AMP at concentrations that caused a maximal increase in either $[Ca^{2+}]_i$ or the generation of inositol phosphates. These results demonstrate that activation of the NMB-preferring Bn receptor does not lead to activation of adenylate cyclase.

The fact that the NMB-preferring Bn receptors identified by the ligand-binding studies were causing the changes in cellular Ca^{2+} and inositol phosphates was supported by the comparison of the abilities of GRP and NMB to alter cell function or occupy NMB-preferring receptors. For increasing both cytosolic Ca^{2+} and inositol phosphate generation, NMB was > 50-fold more potent that GRP, similar to their relative potencies for NMB receptor occupation from the binding studies, suggesting that receptor occupation mediated the changes in biological activity. Even though there was close agreement in the relative affinities of GRP and NMB for occupying the NMB-preferring Bn receptors and for altering cell function, there was an approx. 5-fold higher absolute affinity for either NMB or GRP to alter cell function than to occupy the NMB-preferring receptor. Similar results have been reported with the GRP-preferring receptor as well as with receptors for a number of other gastrointestinal peptides [6,32,58]. This suggests that, as is the case with receptors for these other gastrointestinal peptides, there are spare NMB-preferring Bn receptors on C-6 tumour cells, and that submaximal receptor occupation can cause a maximal change in $[Ca^{2+}]_i$ or inositol phosphates.

In conclusion, the present study demonstrates that C-6 tumour cells possess functional NMB-preferring Bn receptors. The availability of functional receptors for these peptides on this cell line should prove useful for further studies investigating the cellular basis of action of peptides that interact with this receptor with high affinity. Results from studies investigating the changes

in cellular function caused by NMB-related peptides in this tumour cell line should be applicable to the cellular basis of action of peptides at this receptor in the CNS, because cDNA hybridization studies suggest that the same gene encodes for the oesophageal as well as the CNS NMB-preferring Bn receptor and that C-6 tumour cells also express this gene. The present studies showing almost identical affinities for various Bn receptor agonists and antagonists on the C-6 tumour cells and the oesophageal NMB receptor transfected into Balb 3T3 cells further suggests that the C-6 tumour cell NMB receptor and the rat oesophageal receptor are identical. Thus the results from studying the ability of agents to alter cell function by occupying the C-6 NMB-preferring Bn receptor should relate to the ability of the agents to alter cell function in the CNS and other tissues possessing this receptor.

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