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Binding properties of the novel, non-peptide CGRP receptor antagonist radioligand, [^{3}H]BIBN4096BS

Marcus Schindler *, Henri N. Doods

Department of Cardiovascular Research I, Boehringer Ingelheim Pharma KG, Birkendorfer Strasse 65, 88397 Biberach, Germany

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Abstract

BIBN4096BS {[*R*-(*R*,(*R**,*S**)]-*N*-[2-[[5-amino-1-[[4-(4-pyridinyl)-1-piperazinyl]carbonyl] pentyl]amino]-1-[(3,5-dibromo-4-hydroxyphenyl)methyl]-2-oxoethyl]-4-(1,4-dihydro-2-oxo-3(2*H*)-quinazolinyl)-,1-Piperidinecarboxamide} is a selective calcitonin gene-related peptide (CGRP) receptor antagonist with a picomolar affinity to the CGRP receptor in human neuroblastoma SK-N-MC cells. Here, we describe the characterisation of the binding properties of the tritiated radioanalogue of BIBN4096BS in SK-N-MC cells as well as in marmoset tissue. [^{3}H]BIBN4096BS showed reversible and saturable binding to SK-N-MC cells with a K_D of 0.045 nM. In competition experiments, [^{3}H]BIBN4096BS is concentration-dependently displaced from SK-N-MC cell membranes by BIBN4096BS as well as by the endogenous ligand CGRP and its analogues with the rank order of affinity BIBN4096BS > human α-CGRP = human β-CGRP>[Cys(Et)^{2,7}]human α-CGRP>adrenomedullin (high affinity site) = human α-CGRP-(8-37) = human β-CGRP-(8-37)>calcitonin = amylin. In the marmoset cortex, saturable [^{3}H]BIBN4096BS binding was observed with a K_D of 0.077 nM. CGRP showed biphasic competition of [^{3}H]BIBN4096BS binding, whilst BIBN4096BS monophasically displaced its radioanalogue with a K_i of 0.099 nM. These data, using [^{3}H]BIBN4096BS, confirm the high affinity of this novel antagonist for the primate CGRP receptor and demonstrate furthermore that this radioligand is a useful tool to study CGRP receptor pharmacology.

Keywords: CGRP receptor; BIBN4096BS; CGRP receptor antagonist; (Marmoset); SK-N-MC

1. Introduction

Calcitonin gene-related peptide (CGRP) is a 37 amino acid peptide derived from alternative splicing of the calcitonin gene (Amara et al., 1982). CGRP shows a widespread distribution throughout the body (Okimura et al., 1987; Wimalawansa et al., 1987). In keeping with this distribution, a plethora of biological functions for CGRP has been reported. Amongst the most pronounced effects of CGRP are central and peripheral vasodilation (Brain et al., 1986; Wisskirchen et al., 1998; Yoshimoto et al., 1998), cardiac acceleration (Sigrist et al., 1986), inhibition of insulin release from β-cells of the pancreas (Martinez et al., 2000) and reduction of intestinal motility (Fargeas et al., 1985). CGRP is also an important central and peripheral neurotransmitter involved in the pathophysiology of pain: CGRP plays a potentiating modulatory role in the release of substance P (Biella et al., 1991) and has been implicated in the generation of peripheral neurogenic inflammation (Kilo et al., 1997).

CGRP-immunoreactivity is present in trigeminal neurones projecting to the intracranial arteries (O'Connor and van der Kooy, 1988). Stimulation of these neurones leads to vasodilation of cerebral vessels (Edvinsson et al., 1998a). These, together with clinical findings that CGRP-immunoreactivity is elevated in plasma after trigeminal stimulation and during migraine attacks in human subjects (Goadsby et al., 1990, 1998; Goadsby and Edvinsson, 1993) have led to the hypothesis that CGRP is involved in the generation of migraine headache.

CGRP exerts its biological effects by binding to high-affinity G-protein coupled receptors (see Juaneda et al., 2000, for review). High-affinity binding sites have been revealed using receptor binding studies on plasma membranes as well as autoradiographic experiments (Nakamuta et al., 1989; Wimalawansa and El-Kholy, 1993; for review,

* Corresponding author. Tel.: +49-7351-5498232; fax: +49-7351-545128.

E-mail address: marcus.schindler@bc.boehringer-ingelheim.com (M. Schindler).

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see Wimalawansa, 1996). Consistent with the well-documented biological effects, CGRP receptors with high affinity occur in the brain (Henke et al., 1985; Inagaki et al., 1986; Seifert et al., 1985; Yoshizak et al., 1987)—where the highest density of CGRP receptors was found in the cerebellum—the dorsal and ventral spinal cord (Ye et al., 1999), the intima and medial layers of blood vessels (Edvinsson et al., 1998b), and the spleen, which is the peripheral tissue with the highest density of CGRP binding sites (Wimalawansa, 1996).

We have recently described the development of a novel, selective non-peptide CGRP receptor antagonist BIBN4096BS {[*R*-(*R*,(*R**,*S**)]-*N*-[2-[[5-amino-1-[[4-(4-pyridinyl)-1-piperazinyl]carbonyl] pentyl]amino]-1-[(3, 5-dibromo-4-hydroxyphenyl)methyl]-2-oxoethyl]-4-(1, 4-dihydro-2-oxo-3(2*H*)-quinazolinyl)-,1-Piperidinecarboxamide} (Doods et al., 2000). This antagonist has an affinity for CGRP receptors on SK-N-MC human neuroblastoma cells of 14.4 pM and proved to be efficacious in abolishing increases in facial skin blood flow evoked by stimulation of the trigeminal ganglion in the marmoset (Doods et al., 2000). However, the affinity of this compound for CGRP receptors in marmoset tissues is unknown.

The aim of the study presented here was to characterise the binding properties of the newly developed tritiated radioanalogue, [^{3}H]BIBN4096BS to CGRP receptors on human neuroblastoma SK-N-MC cells and in various marmoset tissues.

2. Materials and methods

2.1. Drugs, chemicals and cells

[^{125}I]human α-CGRP was purchased from Amersham (Hannover, Germany); all other peptides were purchased from Neosystems (Strasbourg, France); BIBN4096BS was synthesized by Boehringer Ingelheim Pharma (Biberach, Germany). All other chemicals were of analytical grade. Membrane preparations from SK-N-MC cells were commercially obtained from Receptor Biology (Biotrend, Cologne, Germany).

2.2. Animals

Marmosets of either sex ($n=3$) were killed with an overdose of sodium pentobarbitone. Animals were kept and treated according to the guidelines set by the federal authorities.

2.3. Synthesis of [^{3}H]BIBN4096BS

[^{3}H]BIBN4096BS was prepared in a three-step custom-synthesis by I.I.C.H. (Leawood, USA) in cooperation with the Laboratory of Isotope Chemistry at Boehringer Ingelheim, Biberach. The tritium was introduced into the 3-piperidin-4-yl-3,4-dihydro-1*H*-quinazolin-2-one moiety by catalytic reduction of the precursor 3-pyridin-4-yl-3,4-dihydro-1*H*-quinazolin-2-one with tritium gas. The radiochemical purity was assessed by high-performance liquid chromatography (HPLC) using an Inertsil ODS II column and a gradient from 0.5% KH_2PO_4 (pH 3.0) and acetonitrile as eluent. The identity was confirmed by mass spectrometry and co-chromatography on HPLC.

2.4. Membrane preparation from marmoset tissue

Marmoset brains were dissected on ice into cortex and total brain (which in the following is defined as total brain minus cortex). Furthermore, from the same animals, spleen and dura mater were prepared. Brain tissues and spleen were weighed and were taken up in 10 ml/g wet weight of ice-cold binding buffer (10 mM Tris; 50 mM NaCl, 5 mM $MgCl_2$; 1 mM EDTA; pH 7.4). Samples were then homogenised using an Ultraturrax for 30 s. Dura mater samples were frozen in liquid nitrogen and tissue powder was prepared using a Braun Dismembrator for 2 min. Tissue powder was then taken up as described for the other tissues. All following steps were carried out at 4 °C. The suspension was centrifuged for 20 min at $40{,}000 \times g$, the resulting pellet was then re-homogenised and re-centrifuged as described. The final pellet was then taken up in 10 ml buffer/g wet weight and stirred on ice for 10 min. The suspension was then used directly or frozen in aliquots at − 80 °C. Protein concentration was measured using standard methodology (Bradford, 1976).

2.5. General receptor binding assay

After thawing, the homogenates were diluted 1:200 with binding buffer and thoroughly resuspended. 250 μl of the homogenates were incubated for various times at room temperature with either [^{125}I]human α-CGRP or [^{3}H]BIBN4096BS radioligand and test compound, when applicable, in a total volume of 270 μl. The incubation was terminated by filtration through polyethylene imine (0.1%)-treated GF/B glass fiber filters using a cell harvester. The protein-bound radioactivity was determined in a gamma-counter for [^{125}I]human α-CGRP and in a scintillation counter for [^{3}H]BIBN4096BS. The nonspecific binding was defined as radioactivity bound in the presence of 1 μM human αCGRP during the incubation period.

2.6. [^{3}H]BIBN4096BS saturation binding

After thawing, the homogenates were diluted 1:200 (marmoset cortex 1:50) with binding buffer and thoroughly resuspended. A total of 250 μl of the homogenates were incubated for 180 min at room temperature with increasing concentrations (0.025–5 nM) of [^{3}H]BIBN4096BS in a total volume of 270 μl. The nonspecific binding was defined as

radioactivity bound in the presence of 1 μM BIBN4096BS during the incubation period.

2.7. [^{3}H]BIBN4096BS association kinetic studies

The binding protocol described above with a fixed radioligand concentration of 50 pM was followed except that the incubation times varied from 5 to 1440 min.

2.8. [^{3}H]BIBN4096BS dissociation kinetic studies

The binding protocol described above was followed except that the initial incubation was carried out for 120 min, before a fixed concentration of cold substance, either human α-CGRP (220 nM) or BIBN4096BS (30 nM) was added. The dissociation was followed for various lengths of time (2 min until 1320 min).

2.9. Competition studies

Following the general receptor binding protocol, increasing concentrations of cold reference compounds were added to the reaction mixture containing 50 pM [^{3}H]BIBN4096BS or 50 pM [^{125}I]human α-CGRP and allowed to proceed for 180 min at room temperature.

2.10. Data analysis

The data are presented as the geometrical mean ± S.E.M. of at least three experiments (unless indicated otherwise), carried out in triplicate. To generate IC_{50} or K_i values, data were expressed as percentage of specific binding, with values obtained at the lowest compound diution set at zero and at the highest dilution at 100%, respectively. pIC_{50} and K_D calculations were carried out using non-linear regression analysis using GraphPad Prism™.

3. Results

3.1. Optimisation of assay conditions

Initially, the conditions for the various binding assays (SK-N-MC cells and various marmoset tissues) were optimised. The specific binding of [^{3}H]BIBN4096BS to SK-N-MC membranes was tested in the pH range of pH 6 to pH 9, with best results obtained at pH 7.4 (data not shown). Specific binding was also dependent on the incubation time. At room temperature, specific binding of [^{3}H]BIBN4096BS to SK-N-MC membranes slowly increased and reached a

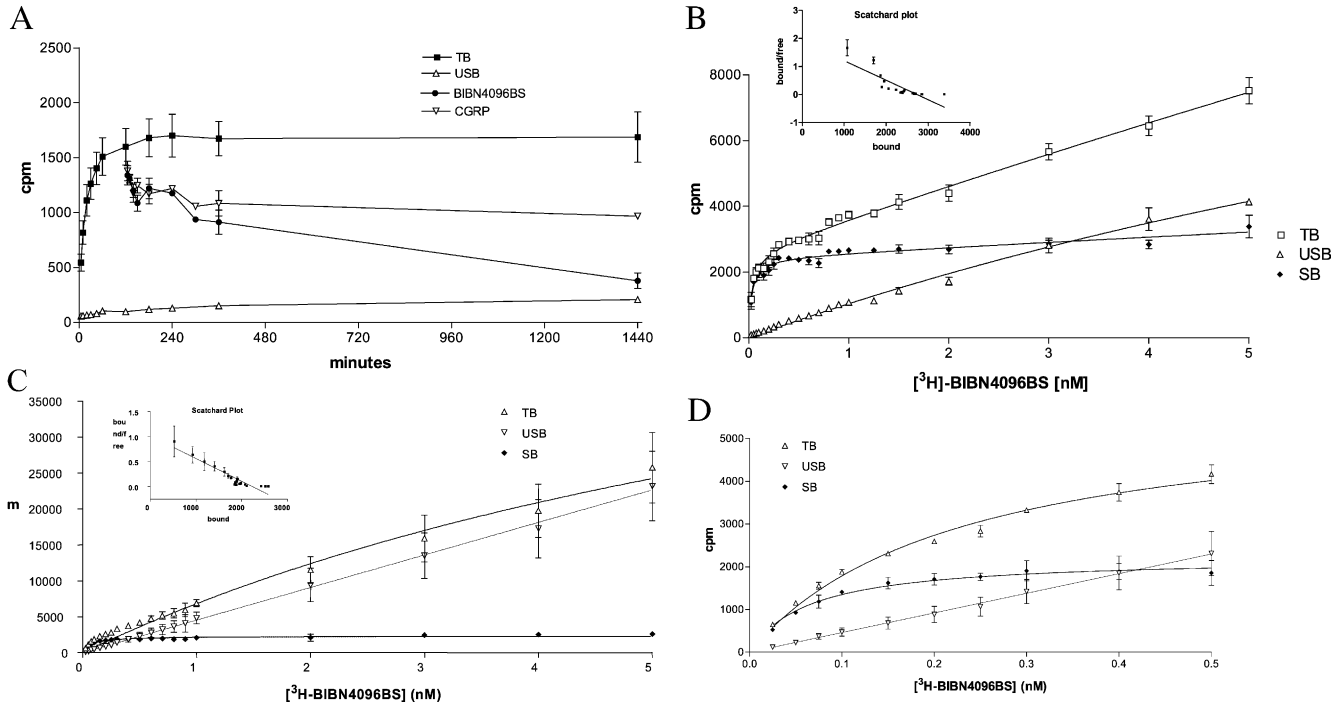

Fig. 1. (A) Association/dissociation studies using [^{3}H]BIBN4096BS on SK-N-MC cell membranes. Membranes were incubated with [^{3}H]BIBN4096BS at RT in the absence (■,TB) or presence (△, USB) of 1 μM BIBN4096BS. After an incubation time of 120 min, an excess of unlabelled BIBN4096BS (●, 30 nM) or human-α CGRP (▽, 200 nM) was added. (B) Saturation isotherm analysis using [^{3}H]BIBN4096BS on SK-N-MC cell membranes. Ligand concentration ranged from 0.025 to 5 nM. Total binding (□) and non-specific binding (△,1 μM h-α-CGRP) both increase, whilst specific binding (◆) reaches a plateau. Insert: Scatchard plot of the same data. (C) Saturation isotherm analysis using [^{3}H]BIBN4096BS on marmoset cortex membranes. Ligand concentration ranged from 0.025 to 5 nM. Total binding (▽) and non-specific binding (△, 1 μM h-α-CGRP) both increase, whilst specific binding (◆) reaches a plateau. Insert: Scatchard plot of the same data. (D) Same as data shown in C, with detailed illustration of the data points from 0 to 0.5 nM radioligand.

Table 1
K_i values for the competition of [^{3}H]BIBN4096BS from SK-N-MC cell membranes for reference compounds and BIBN4096BS

Substance	K_i (high affinity, nM)	K_i (low affinity, nM)
BIBN4096BS	0.07 ± 0.008 (8)	–
Human α-CGRP	0.58 ± 0.09 (7) [39 ± 6%]	48.6 ± 5.3 (8) [60 ± 6%]
Human β-CGRP	0.95 ± 0.21 (5) [46 ± 4%]	17.3 ± 1.6 (5) [54 ± 4%]
[Cys(Et)^{2,7}]human α-CGRP	3.45 ± 2.45 (6) [36 ± 7%]	78.6 ± 17.3 (6) [64 ± 7%]
Adrenomedullin	27.1 ± 2.3 (3) [50 ± 6%]	1227 ± 397 (3) [50 ± 6%]
Human α-CGRP-(8-37)	32.6 ± 6.2 (5)	–
Human β-CGRP-(8-37)	32.1 ± 1.5 (3)	–
hCalcitonin	–	>10,000 (2)
hAmylin	–	>10,000 (2)

Data shown are the mean ± S.E.M. of the number of experiments indicated in brackets, carried out in triplicate. Numbers in square brackets indicate percentage of high and low affinity binding sites, respectively.

plateau at 120 min and beyond. Specific [^{3}H]BIBN4096BS binding remained on a stable plateau for up to 24 h incubation time (Fig. 1A).

3.2. Kinetic studies

Binding of [^{3}H]BIBN4096BS to SK-N-MC cells was found to be saturable after incubation times of 120 min and beyond. After equilibrium was reached, an excess of either human α-CGRP or BIBN4096BS was added (Fig. 1A). This time-dependently displaced [^{3}H]BIBN4096BS from its binding site. After 24 h incubation under these conditions, no specific binding was detected for competition of [^{3}H]BIBN4096BS by BIBN4096BS, whilst human α-CGRP did not completely wash out [^{3}H]BIBN4096BS binding. For the dissociation of [^{3}H]BIBN4096BS, a $t_{1/2}$ of 357 min, with a k_{off} value of 0.0018 min^{-1} was calculated.

3.3. Saturation studies

Saturation isotherm studies using [^{3}H]BIBN4096BS in the range of 0.025–5 nM demonstrated that specific binding to SK-N-MC (Fig. 1B) and marmoset cortex membranes (Fig. 1C) was saturable. Scatchard plots were calculated, from which a K_D of 0.045 nM and a B_{max} of 402 fmol mg^{-1} protein ($n=3$) were determined for [^{3}H]BIBN4096BS binding sites on SK-N-MC and a K_D of 0.077nM and a B_{max} of 0.93 fmol mg^{-1} protein for [^{3}H]BIBN4096BS binding to marmoset cortex membranes ($n=3$).

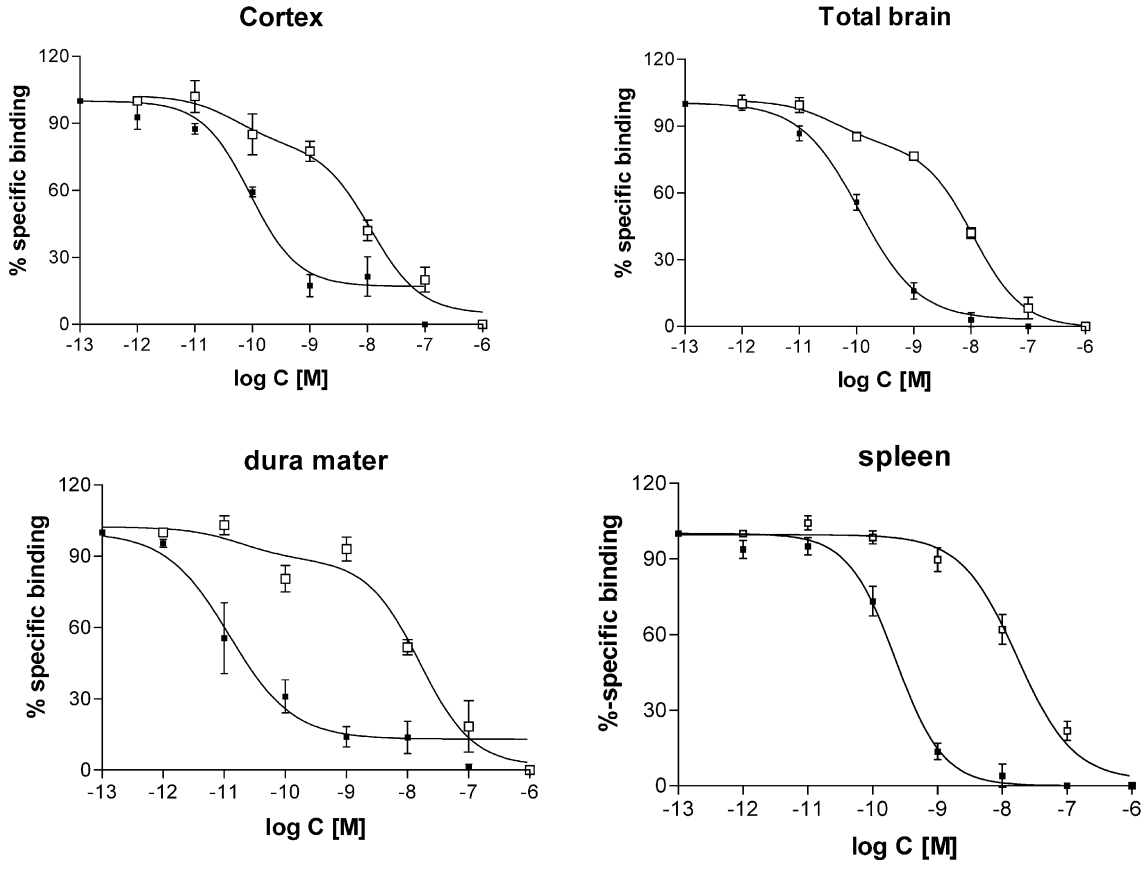

Fig. 2. Competition curves of either BIBN4096BS (■) or human α-CGRP (□) on the binding of [^{3}H]BIBN4096BS to marmoset tissues (regions are indicated). The resulting pIC_{50} values are summarised in Table 2.

Table 2
pIC_{50} and S.E.M. values for the inhibition of specific [^{125}I]CGRP or [^{3}H]BIBN4096 binding, respectively, on the marmoset tissues indicated

Radioligand	[^{125}I]CGRP		n_H	[^{3}H]BIBN4096			n_H
Tissue/cold ligand	CGRP	BIBN4096BS		CGRP	CGRP-(8-37)	BIBN4096BS	
Cortex	9.5 ± 0.07 (5)	10.2 ± 0.07 (5)	− 1.1	10.3/7.9 (4)	7.9 ± 0.07 (3)	10.2 ± 0.08 (4)	− 1.04
Total brain	9.6 ± 0.08 (4)	10.4 ± 0.06 (4)	1.1	10.3/7.9 (4)	n.d.	9.9 ± 0.06 (4)	− 0.84
Dura mater	n.d.	n.d.		10.7/7.8 (4)	n.d.	10.5 ± 0.16 (4)	− 0.84
Spleen	9.9 ± 0.43 (3)	10.0 ± 0.16 (4)		7.8 (4)	6.8 ± 0.08 (3)	9.7 ± 0.06 (4)	− 1.18

Numbers in brackets denote numbers of experiments carried out in triplicate. n.d. = Not determined. n_H = Hill slope.

3.4. Competition studies

A number of CGRP receptor related compounds, agonists and antagonists, were tested to evaluate their potencies in displacing [^{3}H]BIBN4096BS from SK-N-MC membrane binding sites. The K_i values obtained showed the following rank order of affinities: BIBN4096BS > human α-CGRP = human β-CGRP > [Cys(Et)^{2,7}]human α-CGRP > adrenomedullin (high affinity site) = human α-CGRP-(8-37) = human β-CGRP-(8-37) > calcitonin = amylin (summarised in Table 1). Furthermore, in pilot studies, the linear analogue, [Cys(Acm)^{2,7}]human α-CGRP was tested and found to have an IC_{50} value of 437nM (data not shown, $n = 2$).

Furthermore, the IC_{50} values for BIBN4096BS and human α-CGRP were determined in marmoset cortex, total brain, dura mater and spleen. BIBN4096BS showed monophasic competition of both radioligands [^{3}H]BIBN4096BS (Fig. 2) or [^{125}I]human α-CGRP with Hill slopes not significantly different from unity (see Table 2), whilst CGRP displaced [^{125}I]h-α-CGRP monophasically and [^{3}H]BIBN4096BS biphasically (Fig. 2), respectively. The high-affinity component of the biphasic competition curves was abolished after pre-treatment of the membrane suspension with 10 μM GTP (data not shown). The peptide antagonist CGRP-(8-37) displaced [^{3}H]BIBN4096BS monophasically from marmoset cortex and spleen membranes. Both amylin and calcitonin were unable ($pIC_{50} > 5$) to compete for [^{3}H]BIBN4096BS binding sites in marmoset cortex. The pIC_{50} values obtained are summarised in Table 2.

4. Discussion

In this study, we have evaluated the binding properties of the novel CGRP receptor radioligand, [^{3}H]BIBN4096BS. In SK-N-MC cells, a human neuroblastoma cell line, which possesses functional $CGRP_1$ receptors (Muff et al., 1992), [^{3}H]BIBN4096BS showed specific binding. Kinetic studies demonstrated that [^{3}H]BIBN4096BS binding was time-dependent and reached an equilibrium at 120 min. [^{3}H]BIBN4096BS binding could be time-dependently reversed when an excess of cold BIBN4096BS was added to the reaction. In saturation experiments, it was further demonstrated that [^{3}H]BIBN4096BS binding was saturable. Scatchard plot analysis revealed a single, high affinity binding site with an apparent K_D of 45 pM, close to the K_i value found for BIBN4096BS by competition studies (Doods et al., 2000). These data indicate that [^{3}H]BIBN4096BS labels a single high-affinity binding site in neuroblastoma cells. [^{3}H]BIBN4096BS is most potently displaced by its unlabelled analogue, BIBN4096BS, followed by the natural ligands human α-CGRP and β-CGRP being approximately equipotent, [Cys(Et)^{2,7}]human α-CGRP, adrenomedullin (high affinity binding site) and CGRP-(8-37) approximately 40 times less potent. Both, human α-CGRP and β-CGRP showed a biphasic competition of [^{3}H]BIBN4096BS binding, whilst the antagonists BIBN4096BS and CGRP-(8-37), as expected, exhibited monophasic competition curves. Nevertheless, the IC_{50} values determined for the high affinity sites of both agonists correlate well with K_i values obtained in studies using [^{125}I]CGRP as the radioligand, in a variety of tissues (see, e.g. Dumont et al., 1997; Poyner, 1992).

The association of [^{3}H]BIBN4096BS to SK-N-MC membranes is slow and is therefore in keeping with results obtained from using the "natural" ligand [^{125}I]human α-CGRP on this cell line: both radioligands reach an equilibrium of specific binding after an incubation time of 120 min or more (Muff et al., 1992). Furthermore, the dissociation kinetics for either radioligand is also very similar. Only a fraction of [^{3}H]BIBN4096BS binding could be displaced after 120 min, nearly identical to the small proportion (~ 30%) of [^{125}I]human α-CGRP binding removed from SK-N-MC cells after an identical incubation time (Muff et al., 1992). However, we have shown here that [^{3}H]BIBN4096BS binding can be completely displaced by BIBN4096BS when using incubation times for up to 24 h, a condition not tested in the previous publication using [^{125}I]human α-CGRP binding (Muff et al., 1992). A small proportion of specific binding remained, however, even after this long incubation time.

In a previous study, we have demonstrated that BIBN4096BS possesses high affinity for the CGRP receptor on SK-N-MC membranes, whilst the affinity for the rat spleen CGRP receptor was found to be 236-fold less than that in SK-N-MC (Doods et al., 2000). Furthermore, BIBN4096BS showed high potency in a functional in vivo experiment in marmoset. To evaluate whether the high affinity found for the SK-N-MC CGRP receptor was paralleled in the marmoset, we used [^{3}H]BIBN4096BS to investigate its binding properties to membranes from marmoset cerebral cortex, total brain, dura mater and spleen.

[^{3}H]BIBN4096BS binding to marmoset cortex was found to be saturable and with high affinity, apparently to a single binding site, with a K_D similar to that seen in SK-N-MC cells, although B_{max} values differed significantly. Interestingly, the pIC$_{50}$ value found for BIBN4096BS in marmoset spleen was slightly lower than that in the brain tissues (9.7 compared with 10.2), which was paralleled by different pIC$_{50}$ values for the peptide antagonist CGRP-(8-37) (7.9 in cortex compared with 6.8 in spleen). Using [^{125}I]human α-CGRP as the radioligand, however, pIC$_{50}$ values for BIBN4096BS and CGRP did not differ from those obtained in brain (see Table 2). The affinities found in cortical tissue, however, corresponded well with those obtained in SK-N-MC cells. Furthermore, it should be noted that, when using human α-CGRP to displace [^{3}H]BIBN4096BS binding from spleen, only a low affinity component was revealed. Whether these, at least for [^{3}H]BIBN4096BS somewhat subtle, differences may be explained by distinct, tissue-specific CGRP receptor subtypes (Dennis et al., 1990; see also Poyner and Marshall, 2001 for discussion of CGRP receptor heterogeneity) needs to be further evaluated.

In summary, we have shown here that BIBN4096BS and its radioanalogue have high affinity for a CGRP receptor on human neuroblastoma cells as well as marmoset brain and dura mater, the latter being a structure involved in humans in the pathophysiology of migraine headache and thus a potential site of action for BIBN4096BS. Further work comparing functional data with binding studies on the same tissue is needed to fully characterise the CGRP receptor(s) targeted by BIBN4096BS.

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