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Short communication

Labeling of neuropeptide Y receptors in SK-N-MC cells using the novel, nonpeptide Y_1 receptor-selective antagonist [^{3}H]BIBP3226

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Abstract

The binding of tritium-labelled BIBP3226, N^2-(diphenylacetyl)-*N*-[(4-hydroxy-phenyl)methyl]-D-arginine amide, to human neuroblastoma SK-N-MC cells was investigated. [^{3}H]BIBP3226 reversibly binds to neuropeptide Y receptors of the Y_1 subtype expressed in SK-N-MC cells with a K_D of 2.1 ± 0.3 nM (mean ± S.E.M., $n = 3$) and a B_{max} of 58400 ± 1100 sites/cell. Non-specific binding did not exceed 30% of the total radioactivity bound at K_D. In competition experiments [^{3}H]BIBP3226 is concentration-dependently displaced by neuropeptide Y and its peptide analogues with an affinity pattern neuropeptide Y ≈ [Leu^{31},Pro^{34}]neuropeptide Y ≫ neuropeptide Y-(18–36). This rank order of potencies is consistent with the interaction of [^{3}H]BIBP3226 with neuropeptide Y receptors of the Y_1 subtype. Therefore, [^{3}H]BIBP3226 can be used as selective ligand to study neuropeptide Y Y_1 receptors.

Keywords: Neuropeptide Y; SK-N-MC cell; Radioligand binding; BIBP3226

1. Introduction

Neuropeptide Y, a 36 amino acid, C-terminally amidated peptide, was discovered in porcine brain (Tatemoto, 1982) and is one of the most abundant neuropeptides in the mammalian brain and appears to exert a variety of physiological effects such as the control of feeding behavior (Dryden et al., 1994; Stanley et al., 1992) or clinical symptoms of anxiety and depression (Heilig et al., 1993). In addition, neuropeptide Y co-released with noradrenaline from sympathetic nerve endings, is a potent vasoconstrictor on certain blood vessels (for review see Walker et al., 1991). Neuropeptide Y has been characterized as a neuromodulator and neurotransmitter candidate. Receptors for neuropeptide Y have been found on membranes prepared from both central and peripheral tissues (Wahlestedt and Reis, 1993). The pharmacological effects of neuropeptide Y can be attributed to its interaction with at least two different receptor subtypes, termed Y_1 and Y_2. Neuropeptide Y receptors of the Y_1 subtype require the complete amino acid sequence of neuropeptide Y for activation and are characterized by high affinity for the peptide agonists neuropeptide Y, [Leu^{31},Pro^{34}]neuropeptide Y or [Pro^{34}]-neuropeptide Y (Grundemar et al., 1993). In contrast, the neuropeptide Y Y_2 receptor subtype can be stimulated by N-terminally truncated neuropeptide Y-related agonists (e.g neuropeptide Y-(18–36), neuropeptide Y-(13–36).

Recently, we described BIBP3226 (N^2-(diphenylacetyl)-*N*-[(4-hydroxy-phenyl)methyl]-D-arginine amide) as a novel, nonpeptide neuropeptide Y receptor antagonist (Rudolf et al., 1994). The pharmacological properties of this compound include nanomolar affinity for neuropeptide Y Y_1 receptor binding sites as well as high subtype selectivity over neuropeptide Y Y_2 receptors. In addition, the selectivity of the drug for neuropeptide Y receptors was demonstrated in a number of receptor and enzymatic assays (Rudolf et al., 1994). These properties made this compound an attractive choice for radiolabeling. It was expected that the resul-

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tant radioligand could be a useful tool for the study of neuropeptide Y subtype 1 receptors.

2. Materials and methods

2.1. Materials

Human neuroblastoma SK-N-MC cells were obtained from American Tissue Culture Collection (ATCC). BIBP3226 was tritium-labelled by Amersham Buchler (Hannover, Germany). The reaction product was purified at Dr. Karl Thomae by high performance liquid chromatography (Inertsil ODS 5 μm) using a 0.1% aqueous KH_2PO_4 (pH 7.4)–methanol gradient (20–95%) to give a radiochemical purity of $\geq 93\%$. The mean molecular weight of the compound was determined by mass spectroscopy (Blom et al., 1987) and a specific activity of 1.73 TBq/mmol was determined. Neuropeptide Y and related peptides were obtained from Saxon Biochemicals (Hannover, Germany). All other chemicals were of analytical grade.

2.2. Radioligand binding assay

Human neuroblastoma SK-N-MC cells were grown in minimum essential medium (MEM with Earle's salt, 10% (w/v) fetal calf serum, 1 mM sodium pyruvate, 1% (w/v) non-essential amino acids, 4 mM glutamine). Confluent cells were detached with 0.02% (w/v) EDTA in phosphate-buffered saline (1 min incubation) and suspended in 10 ml incubation buffer (MEM/25 mM *N*-[2-hydroxyethyl]piperazine-*N'*-2-ethanesulfonic acid (Hepes), 0.5% (w/v) BSA, 50 μM phenylmethylsulfonyl fluoride, 0.1% (w/v) bacitracin, 3.75 mM $CaCl_2$). After 5 min centrifugation ($150 \times g$) the pellet was recentrifuged and resuspended in incubation buffer. After counting the cells were diluted to give a concentration of 0.6 million cells/ml. 500 μl of this cell suspension were incubated with 0.1–23 nM [^{3}H]-BIBP3226 for saturation experiments or with 1 nM [^{3}H]BIBP3226 and increasing concentrations of test compounds for 2 h at room temperature in a total volume of 520 μl. 12–14 concentrations of competitor were chosen to bracket the expected IC_{50}. Particle-bound radioligand was assayed by liquid scintillation counting after rapid filtration through polyethyleneimine treated GF/B glass fiber filters using a Skatron cell harvester. Non-specific binding was defined as radioactivity bound in the presence of 1 μM BIBP3226. To distinguish surface-bound from intracellular radioactivity, [^{3}H]BIBP3226 bound to the cell surface was determined using the acid wash technique. After preincubation with radioligand the cells were centrifuged ($250 \times g$, 5 min) and resuspended in either 0.5 ml of incubation buffer or 0.2 M acetic acid. The cells were incubated for 10 min at 0° C. After centrifugation and washing, the cell pellet was lysed in 0.5 ml 1 M NaOH, and the residual radioactivity was determined.

2.3. Data analysis

Saturation and competition data were analyzed by a computer-assisted non-linear least-square curve fitting method by using the RS/1 software package (BBN Research Systems, Cambridge, MA, USA). IC_{50} values were corrected for the radioligand occupancy shift to obtain the inhibition constants (K_i) according to Cheng and Prusoff (1973). Saturation experiments were analyzed according to Scatchard (1949). All data are given as mean ± S.E.M of independent experiments performed in triplicates. Statistical analyses were performed by two-way analysis of variance followed by two-tailed Student's *t*-test. Differences were considered statistically significant at $P \leq 0.05$.

3. Results

The binding of [^{3}H]BIBP3226 (1 nM) to intact human neuroblastoma SK-N-MC cells increased linearly with the cell number (0.1–1.0 million cells/assay tube). The binding of [^{3}H]BIBP3226 to human neuroblastoma SK-N-MC cells was saturable as illustrated in Fig. 1. Non-specific binding did not exceed 30% of the radioligand bound at K_D. Transformation of the saturation binding data indicated that [^{3}H]BIBP3226 bound with high affinity to a single population of cellular receptor sites. The K_D determined was 2.1 ± 0.3 nM (n = 3). The number of receptors labelled by [^{3}H]BIBP3226 was 58400 ± 1100 sites/cell. Specific

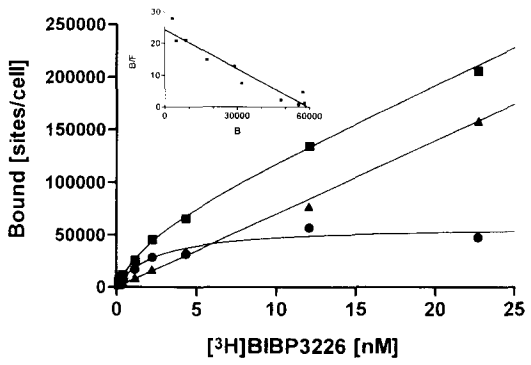

Fig. 1. Saturation isotherm of [^{3}H]BIBP3226 binding to human neuroblastoma cells (SK-N-MC) as described in Materials and methods. Total (squares), specific (circles) and non-specific binding (triangles) was determined for 2 h at room temperature. Non-specific binding was defined in the presence of 1 μM neuropeptide Y. Values are the means of a typical experiment (n = 3) performed in triplicate.

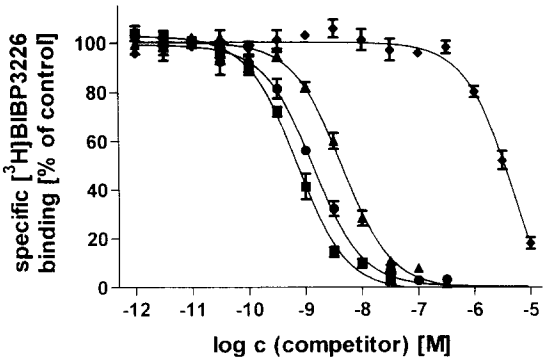

Fig. 2. Inhibition of [^{3}H]BIBP3226 (1 nM) binding to neuropeptide Y receptors on human neuroblastoma cells (SK-N-MC) by neuropeptide Y (squares), [Leu^{31},Pro^{34}]neuropeptide Y (circles), BIBP3226 (triangles) and neuropeptide Y-(18–36) (diamonds) as defined in radioligand displacement studies. Each data point represents the mean of three individual determinations performed in triplicate.

binding was reversible over time upon the addition of unlabelled drug (data not shown) and was undetectable between 1 and 2 h thereafter. The extracellular location of bound [^{3}H]BIBP3226 can be demonstrated by the displacement of bound radioligand by acid treatment of the cells. The total binding after washing the cells with 0.2 M acetic acid (17.1 ± 0.3% of control; $n = 3$) was not significantly different from nonspecific binding obtained in the presence of 1 μM BIBP3226 (16.2 ± 0.5% of control; $n = 3$).

In competition experiments unlabelled BIBP3226, neuropeptide Y and subtype-selective peptide analogues were examined for their ability to displace specific [^{3}H]BIBP3226 binding to human neuroblastoma SK-N-MC cells (Fig. 2). BIBP3226, neuropeptide Y and [Leu^{31},Pro^{34}]neuropeptide Y completely displaced the bound radioligand. Neuropeptide Y was the most potent inhibitor of [^{3}H]BIBP3226 binding with an inhibition constant (K_i) of 0.57 ± 0.07 nM ($n = 3$). The Hill coefficient of the displacement curve (1.08 ± 0.12) was not significantly different from unity, demonstrating the interaction with a single population of binding sites. Specific [^{3}H]BIBP3226 binding was potently inhibited by the Y_1-selective peptide agonist [Leu^{31}, Pro^{34}]neuropeptide Y with a K_i of 0.88 ± 0.08 nM ($n = 3$). Analysis of the displacement curve revealed a Hill coefficient of 0.94 ± 0.07. Unlabelled BIBP3226 inhibited specific radioligand binding with a K_i of 2.7 ± 0.2 nM ($n = 3$) in a monophasic manner (Hill coefficient 1.04 ± 0.02). In contrast, the Y_2-selective agonist neuropeptide Y-(18–36) did not displace radiolabelled BIBP3226 in the nanomolar range and gave a K_i value of 2850 ± 230 nM ($n = 3$).

4. Discussion

In the present study we have characterized the specific binding of [^{3}H]BIBP3226 to human neuroblastoma, SK-N-MC, cells. The binding of the radioligand was reversible and saturable. Acid treatment of the cells after incubation with [^{3}H]BIBP3226 revealed the specific binding of the radioligand occurred solely to binding sites at the extracellular cell surface. The equilibrium dissociation constant (K_D) obtained in saturation experiments is in close agreement with the affinity of BIBP3226 for neuropeptide Y Y_1 receptors as determined earlier (Rudolf et al., 1994) from indirect inhibition studies using the unlabelled drug. The number of binding sites on human neuroblastoma SK-N-MC cells, 58400 sites/cell corresponding to 97 fmol/10^6 cells, labelled by [^{3}H]BIBP3226 was identical to that obtained using the peptide agonist ligand [^{125}I]neuropeptide Y (Feth et al., 1991).

Specific [^{3}H]BIBP3226 binding to intact human neuroblastoma SK-N-MC cells was concentration-dependently inhibited by peptide neuropeptide Y ligands and satisfied the criteria for the interaction with neuropeptide Y receptors. The rank order of potencies for these peptides as determined in this study is neuropeptide Y ≃ [Leu^{31},Pro^{34}]neuropeptide Y ≫ neuropeptide Y-(18–36). The K_i values obtained are in accordance with those reported for the interaction of the peptides with neuropeptide Y Y_1 receptors (Beck-Sickinger and Jung, 1995; Wieland, personal communication) and clearly different from those obtained for Y_2 binding sites in other human cell lines, e.g. SMS-KAN cells.

In conclusion, the results of the present study demonstrate that the selective antagonist radioligand [^{3}H]BIBP3226 can be successfully used to label and study neuropeptide Y subtype 1 receptors. These results, as well as our data with unlabelled BIBP3226 (Rudolf et al., 1994), suggest that the radioligand [^{3}H]BIBP3226, due to its high affinity and selectivity, will be extremely useful in the further classifiaction of neuropeptide Y receptors.

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