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Human neuropeptide YY_1 receptors exert unequal control of the extracellular acidification rate in different cell lines

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Abstract

The ability of the human neuropeptide YY_1 receptor subtype to increase the extracellular acidification rate in different cell lines was investigated by using the Cytosensor Microphysiometer. In CHO-Y_1 cells (Chinese Hamster Ovary cells expressing the cloned human neuropeptide YY_1 receptor), neuropeptide Y increased the acidification rate by up to 15% of the basal level with a $-\text{Log}(EC_{50})$ of 7.42. As expected for neuropeptide YY_1 receptors, this response was potently inhibited by the neuropeptide YY_1-selective non-peptide antagonist BIBP3226 ((*R*)-N^2-(diphenylacetyl)-*N*-[(4-hydroxy-phenyl)methyl]-D-arginine amide). Its enantiomer BIBP3435 ((*S*)-N^2-(diphenylacetyl)-*N*-[(4-hydroxy-phenyl)methyl]-D-argininamide) was less potent. The antagonists themselves did not affect the extracellular acidification rate at concentrations up to 10 μM. In SK-N-MC cells (a neuroblastoma cell line of human origin that expresses the neuropeptide YY_1 receptor) no change of the acidification rate could be observed in the presence of neuropeptide Y at concentrations up to 1 μM. For control, the neuropeptide YY_1 receptors were also investigated by assessing whole cell radioligand binding and, at the functional level, by assessing their ability to decrease the forskolin-induced accumulation of cAMP. The specific (i.e., neuropeptide Y-displaceable) binding of [^{3}H]neuropeptide Y was to a homogenous class of high-affinity sites in both SK-N-MC and CHO-Y_1 cells. The equilibrium dissociation constants for [^{3}H]neuropeptide Y, the total number of binding sites and the kinetic constants for association and for dissociation were similar. Neuropeptide Y produced a dose-dependent inhibition of forskolin-induced cAMP accumulation in SK-N-MC cells ($-\log(EC_{50}) = 9.40$) but it did not affect cAMP accumulation in CHO-Y_1 cells. Non-transfected CHO-K1 cells were used as negative control throughout the study. No binding or response could be observed in these cells. Our data suggest that the signalling mechanisms of neuropeptide YY_1 receptors are closely related to the cell type in which they are expressed. © 1998 Elsevier Science B.V.

Keywords: Neuropeptide YY_1 receptor; SK-N-MC neuroblastoma cell; CHO cell; cyclic AMP; Cytosensor microphysiometer; Extracellular acidification; Neuropeptide Y, human

1. Introduction

The neurotransmitter neuropeptide Y is a member of the pancreatic polypeptide family which also includes peptide YY and pancreatic polypeptide (Tatemoto and Mutt, 1980; Tatemoto et al., 1982; Michel, 1991; Gehlert, 1994). Neuropeptide Y is released by both central and peripheral neurons. It is highly abundant in the central nervous system, where it contributes to the control of a wide variety of functions (Stanley and Leibowitz, 1985; Stanley et al., 1992; Dryden et al., 1994; O'Donohue et al., 1985). In the peripheral nervous system, neuropeptide Y is associated with sympathetic vascular control and with the release of catecholamines (Walker et al., 1991; Grundemar and Håkanson, 1993).

Neuropeptide Y stimulates different receptor subtypes which belong to the G protein-coupled, seven-transmembrane domain receptor family. Until very recently, neuropeptide YY_1, neuropeptide YY_2 and neuropeptide YY_3 receptor subtypes could be discriminated by radioligand binding and by functional assays based on differences in their pharmacological profile (Wahlestedt et al., 1992). Molecular cloning experiments have evidenced the existence of additional receptor subtypes (i.e., PP1, neuropeptide YY_4 and neuropeptide YY_5 receptors) (Larhammar et

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al., 1992; Bard et al., 1995; Lundell et al., 1995; Gerald et al., 1995).

The neuropeptide YY_1 receptor subtype mediates vascular smooth muscle contraction and potentiates the pressor response to other vasoconstrictors (Walker et al., 1991; Wahlestedt and Reis, 1993; Grundemar and Håkanson, 1994; Evequoz et al., 1994). Because of the prominent role of this receptor subtype in cardiovascular function, much effort has already been spent in the development of neuropeptide YY_1-selective antagonists and a number of potent low-molecular-weight nonpeptidic compounds (BIBP3226: ((*R*)-N^2-(diphenylacetyl)-*N*-[(4-hydroxyphenyl)methyl]-D-arginine amide), SR120107A: (1-[2-[2-(2-naphthylsulphamoyl)-3-phenylpropionamido]-3-[4[*N*-[4-(dimethylaminomethyl) *-trans-* cyclohexylmethyl]amidino] phenyl]propionyl]-pyrrolidine, (*R*,*R*) stereoisomer) and SR120819A: (1-[2-[2-(-naphthylsulfamoyl)-*cis*-cyclohexylmethyl]amidino]-phenyl]propionyl]pyrrolidine)) have recently been synthesised (Rudolf et al., 1994; Serradeil-Le Gal et al., 1994, 1995; Lundberg et al., 1996). So far, neuropeptide YY_1 receptors have been shown to affect at least two independent second messenger systems in cell lines, leading to the release of calcium from intracellular stores and to a decrease in the intracellular cAMP concentration (Feth et al., 1991; Herzog et al., 1992; Motulsky and Michel, 1988; Shigeri and Fujimoto, 1992).

When transfected into suitable host cells, several G-protein-coupled receptors, such as muscarinic M_1, β_2-adrenoceptor, prostaglandin E and dopamine receptors (Owicki et al., 1990; McConnell et al., 1991; Neve et al., 1992; Chio et al., 1993), have been demonstrated to increase the rate at which cells acidify their environment with products of their energy metabolism (McConnell et al., 1992). This phenomenon can conveniently be monitored with the newly developed Cytosensor® Microphysiometer, which makes use of a light-sensitive potentiometric sensor to measure the rate at which cells affect the pH of their environment. The alleged capability of this method to monitor the effects of agonists and antagonists at the functional level, and this irrespective of intermediate cellular events (McConnell et al., 1992), prompted us to determine whether it can be used to investigate neuropeptide YY_1 receptors in cell lines.

We compared two cell lines which contain the human neuropeptide YY_1 receptor subtype: one of human origin (the SK-N-MC cells, a human neuroblastoma cell line that endogenously expresses the neuropeptide YY_1 receptor; Wahlestedt et al., 1992; Shigeri and Fujimoto, 1992) and the other of animal origin transfected with the gene coding for the human neuropeptide YY_1 receptor (i.e., CHO-Y_1 cells: Chinese Hamster Ovary cells expressing the cloned human neuropeptide YY_1 receptor; Herzog et al., 1992). Here, we show for the first time the ability of neuropeptide Y to increase the extracellular acidification rate in CHO-Y_1 cells. Although no response was observed with SK-N-MC cells, the presence of functional neuropeptide YY_1 receptors on these cells was demonstrated by radioligand binding and by their ability to decrease the forskolin-induced accumulation of cAMP.

2. Materials and methods

2.1. Materials

N-[propionyl-^{3}H] neuropeptide Y ([^{3}H]neuropeptide Y) (80 Ci/mmol) was obtained from Amersham (Little Chalfont, UK). Neuropeptide Y (neuropeptide Y, human) was from Cambridge Research Biochemicals (UK). Bovine serum albumin (Fraction V) was from Sigma (St. Louis, MO, USA). BIBP3226 ((*R*)-N^2-(diphenylacetyl)-*N*-[(4-hydroxy-phenyl)methyl]-D-argininamide) and BIBP3435 ((*S*)-N^2-(diphenylacetyl)-*N*-[(4-hydroxy-phenyl)methyl]-D-argininamide) were from Albany Molecular Research (Albany, NY, USA). These antagonists were dissolved in dimethylsulphoxide (DMSO) to obtain stock solutions of 20 mM and further diluted in the incubation media to obtain the appropriate concentrations. Bacitracin (60 units/mg) and 3-*iso*butyl-1-methyl-xanthine (IBMX) were from Acros Organics (Beerse, Belgium) and forskolin was from Sigma. All other chemicals were of the highest grade commercially available. Human neuroblastoma SK-N-MC cells were obtained from the American Tissue Culture Collection. Wild-type cells (CHO-K1) were kindly donated by Dr. H. Verschueren (Pasteur Institute, Brussels, Belgium) and CHO cells stably expressing the human Neurpeptide YY_1 receptor (denoted as CHO-Y_1 cells, Herzog et al., 1992) were obtained from Astra Hässle (Molndal, Sweden).

2.2. Cell culture

SK-N-MC and CHO-Y_1 cells were cultured in 75-cm^2 flasks in Dulbecco's Modified Essential Medium (DMEM), supplemented with L-glutamine (2 mM), 2% of a stock solution containing 5000 I.U./ml penicillin and 5000 μg/ml streptomycin (Gibco), 1% of a solution MEM containing non-essential amino acids (Gibco), 1 mM sodium pyruvate (Gibco) and 10% fetal calf serum (Gibco). The cells were grown in an atmosphere of 5% CO_2 at 37°C until confluency was reached.

2.3. Radioligand binding

Cells were plated in 24-well plates and cultured until confluency. When confluent, the culture medium was aspirated and 0.5 ml of Dulbecco's PBS buffer (Phosphate-buffered saline, containing 0.132 g/l $CaCl_2 \cdot 2H_2O$, 0.2 g/l KCl, 0.2 g/l KH_2PO_4, 0.1 g/l $MgCl_2 \cdot 6H_2O$, 8 g/l NaCl and 1.44 g/l $Na_2HPO_4 \cdot 2H_2O$) at room temperature was added to each well. This washing procedure was repeated once. After removal of the medium, 400 μl binding buffer (DMEM containing 0.5% bovine serum albumin, 0.1% bacitracin and 0.1 mM phenylmethylsulfo-

nyl fluoride) was added. The plate was left for 30 min at room temperature, after which 50 μl of unlabelled competitor (for competition binding assays), buffer (for total binding) or 0.1 (M (final concentration) of unlabelled neuropeptide Y (for non-specific binding) was added. Subsequently 50 μl of buffer containing [^{3}H]-neuropeptide Y was added and the plates were incubated at 37°C for 90 min. At the end of the incubation, the 24-well plates were placed on ice and the cells were washed three times with Dulbecco's PBS buffer at 4°C as described above. After the last aspiration of buffer, 500 μl of a 1% w/v Triton X-100 solution was added to each well and the plates were further incubated for 1 h at room temperature. Then 400 μl of the solution was transferred to a scintillation vial, 3 ml of scintillation liquid (Optisafe of Wallac, Turku, Finland) was added and the vials were counted for 3 min in a liquid scintillation counter. Final [^{3}H]neuropeptide Y concentrations were 0.5 nM for kinetic studies and 1 nM for competition binding experiments. Concentrations ranged between 0.25 and 5 nM for saturation binding experiments.

Non-specific binding was subtracted from the total binding to yield specific binding. Experiments comprise triplicate determinations and were performed three to six times. The calculation of the binding parameters from the association and dissociation binding curves (k_{obs} and k_2 values), IC_{50} values (concentration of unlabelled drug causing half maximal inhibition) for competition binding curves and K_d (equilibrium dissociation constant for radioligand binding) and B_{max} values (total amount of binding sites) for saturation binding curves was performed by non-linear regression analysis using GraphPad Prism.

2.4. cAMP determination

As for the binding assays, cells were plated in 24 well plates until a monolayer was reached. Before the experiment, cells were washed at room temperature once with Dulbecco's PBS buffer (composition as described above) and once with cAMP assay buffer containing 10 mM HEPES (2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid), 150 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$ and 10 mg/ml bovine serum albumin at pH 7.4. Cells were incubated for 10 min at 37°C with assay buffer (1 ml/well) and then for 10 min at 37°C with assay buffer (400 μl/well) containing 0.5 mM IBMX. To start the experiment, 50 μl assay buffer alone (for basal cAMP accumulation) or containing neuropeptide Y and/or the antagonists (BIBP3226 or BIBP3435) was added. Immediately thereafter, 50 μl assay buffer containing forskolin at a final concentration of 10 μM was added. After incubation for 20 min at 37°C, the buffer was aspirated and 500 μl of ethanol was added to stop the reaction. After a 5-min incubation at room temperature, the fluid was transferred to Eppendorf tubes. The last step was repeated and the ethanol was evaporated at 40°C. The samples were then dissolved in 25 mM Tris–HCl (pH 7.5) containing 4 mM EDTA and the amount of cAMP was determined according to Tovey et al. (1974). Each experiment comprised triplicate determinations.

2.5. Extracellular acidification rate determination

Extracellular acidification rates were measured, using a Cytosensor microphysiometer (Molecular Devices, Menlo Park, CA). Cells were seeded 16–18 h before the experiment into 12-mm diameter disposable polycarbonate cell capsule cups (Molecular Devices) at 3×10^5 cells/cup and incubated at 37°C in 5% CO_2 in growth medium (DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 (g/ml streptomycin). The capsule cups were loaded into the sensor chambers of the microphysiometer and perfused with running medium (growth medium without serum and $NaHCO_3$) at a flow rate of 150 μl/min. This running medium also contained drugs as indicated. Pump cycles (90 s each) were controlled by the Cytosoft software running on a Macintosh computer. For each cycle, cells were perfused with medium for the first 60 s and the pump was switched off for the remaining 30 s. The pH of the medium was recorded during the 65–90-s interval. The acidification rate of the buffer was calculated by the Cytosoft program and rates are reported as % of control, i.e., as % of the average acidification rate during the 4 cycles immediately preceding the addition of neuropeptide Y and/or the antagonists.

3. Results

3.1. Effect of neuropeptide Y receptor stimulation on the release of acidic metabolites

The rate of extracellular acidification by SK-N-MC, CHO-Y_1 and CHO-K1 cells was determined by using the Cytosensor Microphysiometer at 37°C, as described in Section 2. Variations in the amplitude of the extracellular acidification rate in the presence of different concentrations of neuropeptide Y and the kinetics of this cellular response in CHO-Y_1 cells are shown in Fig. 1. The onset of this response was rapid; a maximal increase in acidification rate was observed during the first cycles (of 90 s) in which the cells were perfused with neuropeptide Y. This effect was transient and it took about 20 min for the cells to gain background levels again. The maximal increase, which was measured during either the first or the second cycle, was used to quantify the effect of neuropeptide Y in the ensuing experiments. This increase is expressed as the % increase above each individual basal level preceding cell stimulation.

Neuropeptide Y increased the acidification rate by CHO-Y_1 cells dose dependently until a plateau level was

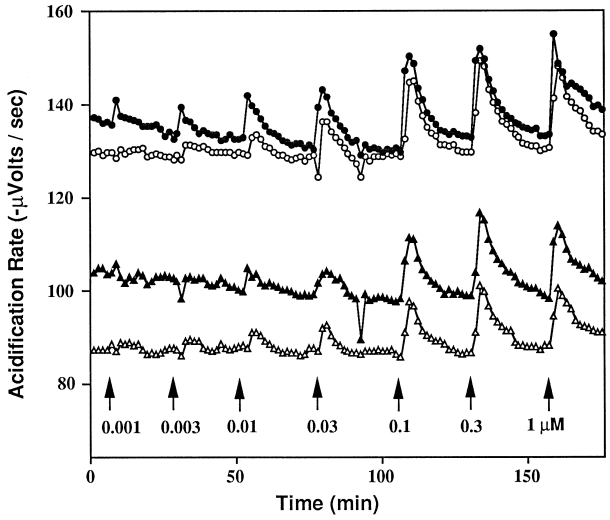

Fig. 1. Neuropeptide Y-dependent increase in extracellular acidification by CHO-Y_1 cells. Each symbol represents the measured rate of acidification corresponding to a 90-s cycle as described in Section 2. Arrows correspond to the time at which cell perfusion with the indicated neuropeptide Y concentrations was initiated. This figure shows four representative tracings obtained in parallel experiments.

attained at about 0.3 μM neuropeptide Y (Fig. 2). The $-\log(EC_{50})$ of neuropeptide Y was 7.42 ± 0.12 ($n = 16$). In contrast, neuropeptide Y (at concentrations up to 1000 nM) did not perceptibly affect the acidification rate by SK-N-MC cells (Fig. 2) or by wild-type CHO-K1 cells (Fig. 3A,C) under the same experimental conditions.

Inhibition experiments with the neuropeptide YY_1-selective antagonist BIBP3226 and with its less active enantiomer BIBP3435 were performed to further ascertain the involvement of neuropeptide YY_1 receptors in the response

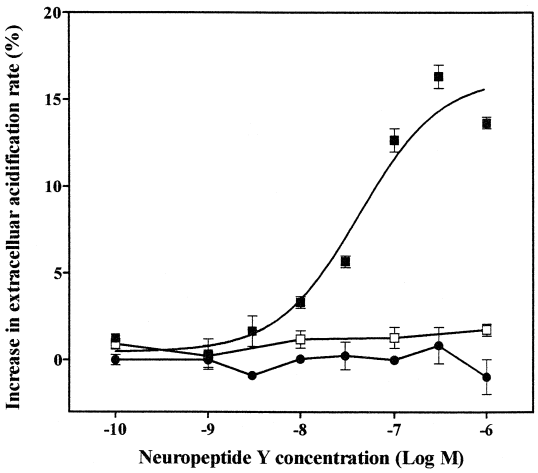

Fig. 2. Dose-response curves for neuropeptide Y-mediated changes in extracellular acidification measured for SK-N-MC cells (●, $n = 4$), CHO-K1 cells (□, $n = 7$) and CHO-Y_1 cells (■, $n = 16$). The peak change in acidification rate was measured at each concentration of NPY and is expressed as % increase above the pre-perfusion level. Each point is the mean ± S.E.M. of the indicated number of independent experiments.

of the CHO-Y_1 cells (Fig. 3A,C). In these experiments the same cells were perfused consecutively with medium containing 100 nM neuropeptide Y in the presence of progressively increasing concentrations of antagonist. In CHO-Y_1 cells, both antagonists produced a dose-dependent inhibition of the neuropeptide Y effect, and BIBP3226 ($-\log(IC_{50}) = 8.05 \pm 0.19$) was more potent than BIBP3435 ($-\log(IC_{50}) = 6.94 \pm 0.47$). Neither antagonist alone was able to affect the basal acidification rate at concentrations up to 10 μM (Fig. 3B,D).

To evaluate potential desensitization of the response, we examined the effect of repeated applications of 100 nM neuropeptide Y alone on the extracellular acidification rate of CHO-Y_1 cells. After several consecutive neuropeptide Y administrations, the magnitude of the response was the same as for unexposed cells.

3.2. Effect of neuropeptide Y receptor stimulation on the cAMP concentration

The basal (i.e., unstimulated) cAMP levels were very low in plated SK-N-MC and CHO-Y_1 cells (about 50 and 2

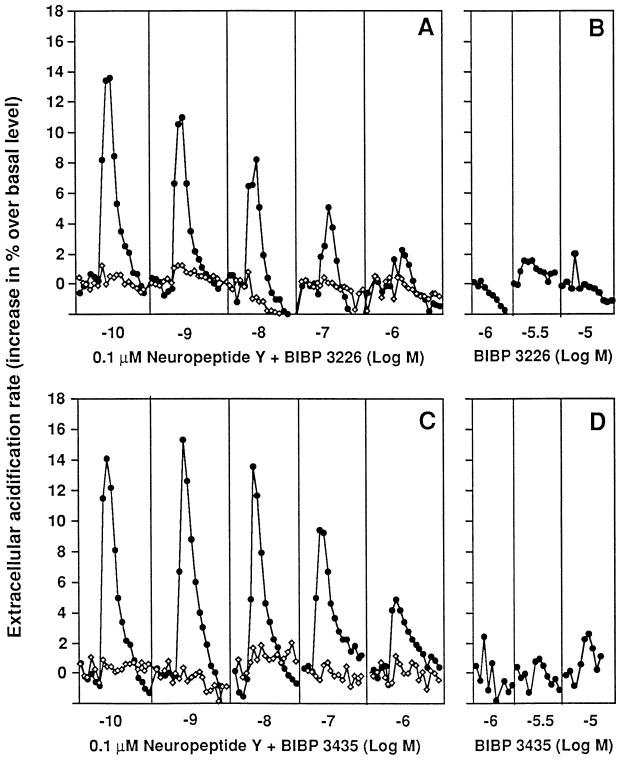

Fig. 3. The effect of BIBP3226 and BIBP3435 on the extracellular acidification rate. Inhibition of the 100 nM neuropeptide Y-stimulated increase in extracellular acidification rate by successive application of increasing concentrations of BIBP3226 (panel A) or its enantiomer BIBP3435 (panel C) to CHO-Y_1 cells (●) and CHO-K1 cells (○). The increase in extracellular acidification rate is expressed as % increase above the pre-perfusion level. BIBP3226 (panel B) BIBP3435 (panel D) alone had no effect on the extracellular acidification rate. Each point is the mean of four experiments.

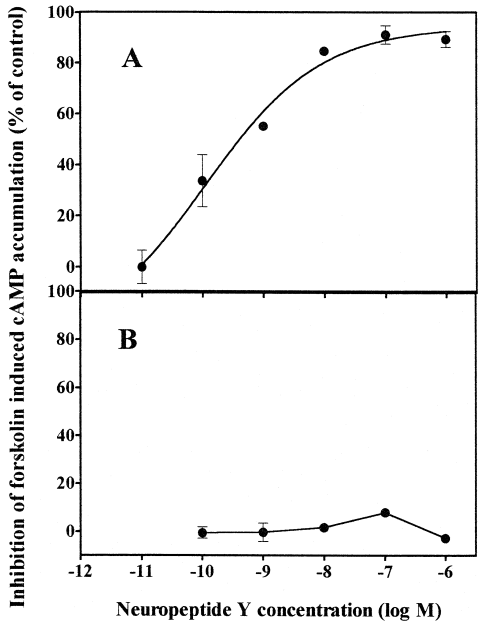

Fig. 4. The inhibition of forskolin-induced cAMP accumulation by neuropeptide Y in SK-N-MC cells (A) and CHO-Y_1 cells (B). Cells were exposed to 10 μM forskolin and increasing concentrations of neuropeptide Y. The inhibitory effect of neuropeptide Y is expressed as the percentage of the cAMP accumulation obtained in the presence of 10 μM forskolin alone (control). The corresponding EC_{50} value for neuropeptide Y in SK-N-MC cells is given in Section 3.

pmol/well, respectively). Incubation of these cell lines with 10 μM forskolin for 20 min at 37°C produced a dramatic increase in the cAMP concentration in both cases (cAMP concentration = 651 ± 48 pmol/well and 309 ± 25 pmol/well, $n = 3$, respectively). Neuropeptide Y inhibited the forskolin-induced cAMP accumulation in a dose-dependent manner in SK-N-MC cells (Fig. 5A) but it did not affect the forskolin-induced cAMP accumulation in CHO-Y_1 cells at concentrations up to 1 μM (Fig. 5B). For SK-N-MC cells, the $-\log(EC_{50})$ of neuropeptide Y was 9.40 ± 0.16 and inhibition was nearly complete at 10 nM neuropeptide Y. This inhibitory action of 10 nM neuropeptide Y was dose dependently relieved by BIBP3226 with a $-\log(IC_{50})$ of 6.24 ± 0.05 (Fig. 6). BIBP3435 was much less potent, and did not affect the inhibition at concentrations up to 1 μM (Fig. 6). 10 μM of BIBP3226 or BIBP3435 alone did not affect basal or forskolin-stimulated cAMP accumulation (not shown), indicating that they had full antagonist properties.

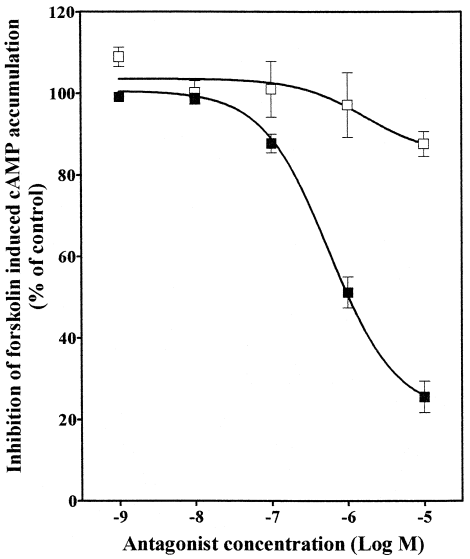

Fig. 5. The effect of BIBP3226 (■) and BIBP3435 (□) on the neuropeptide Y (10 nM) mediated inhibition of the forskolin-induced accumulation of cAMP in SK-N-MC cells. Data are presented as percentages of the inhibition produced by 10 nM neuropeptide Y (control).

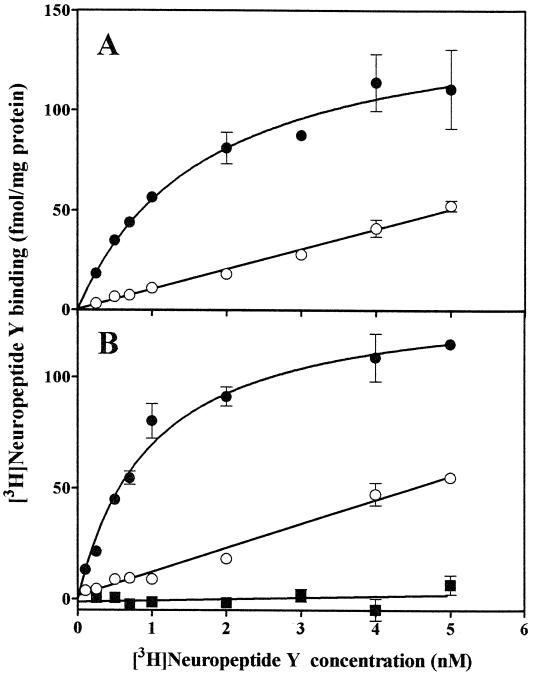

Fig. 6. The saturation binding of [^{3}H]neuropeptide Y to intact SK-N-MC cells (A), intact CHO-Y_1 cells and intact non-transfected CHO-K1 cells (B). Curves show represent specific binding of [^{3}H]neuropeptide Y to SK-N-MC cells (●, A), CHO-Y_1 cells (●, B) and CHO-K1 cells (■, B) and non-specific binding (in the presence of 0.1 mM unlabelled neuropeptide Y) to SK-N-MC cells (○, A) and CHO-Y_1 cells (○, B). The corresponding equilibrium dissociation constants and B_{max} values are listed in Section 3.

3.3. Characterization of neuropeptide Y receptors by radioligand binding

Saturation binding experiments, shown in Fig. 7, revealed that the specific (i.e., neuropeptide Y-displaceable) binding of [^{3}H]neuropeptide Y occurred to an apparently homogeneous class of high-affinity sites on SK-N-MC and CHO-Y_1 cells grown as a monolayer (Hill coefficient (n_H) = 1.00 ± 0.02 and 0.99 ± 0.03, respectively, $n = 3$). The calculated equilibrium dissociation constants (K_d values, 1.92 ± 0.86 and 1.83 ± 0.51 nM, respectively) and the density of sites (B_{max} values, 163 ± 11.3 and 188 ± 5.5

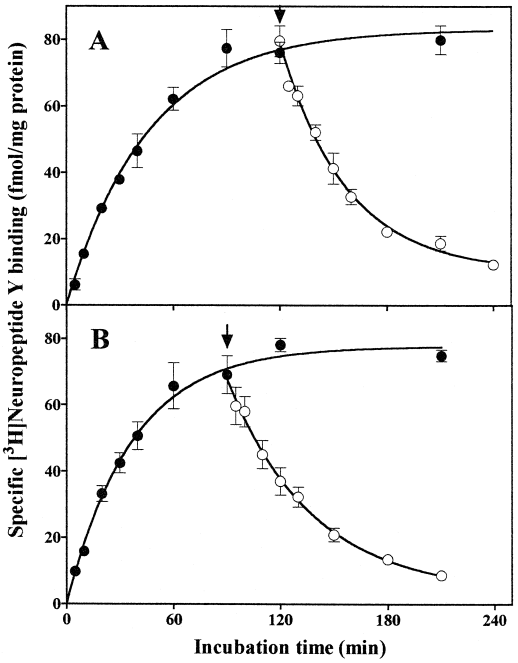

Fig. 7. The time-course of association and dissociation of 0.5 nM [^{3}H]neuropeptide Y from SK-N-MC cells (A) and CHO-Y_1 cells (B). For the association binding (●), specific [^{3}H]neuropeptide Y binding was determined after different incubation times. For the dissociation binding (○), cells were incubated for 120 min (A) or 90 min (B) with [^{3}H]-neuropeptide Y, after which dissociation was initiated by addition of 0.1 mM unlabelled neuropeptide Y (arrow). The corresponding association and dissociation rate constants are given in Section 3.

fmol/mg protein, respectively) were comparable for both cell lines. No specific binding was observed for the wild type CHO-K1 cells.

Kinetic experiments indicated that the specific binding of 0.5 nM [^{3}H]-neuropeptide Y to plated SK-N-MC and CHO-Y_1 cells increased as a function of time until equilibrium was attained after approximately 90 min (Fig. 2). The calculated pseudo-first-order rate constants for association (k_{obs}) at 37°C were 0.021 ± 0.002 min^{-1} and 0.034 ± 0.002 min^{-1}, respectively. Dissociation of [^{3}H]neuropeptide Y from the receptor was initiated by addition of 0.1 μM unlabelled neuropeptide Y to an equilibrated mixture of cells and the radioligand (Fig. 8). The calculated first-order rate constants (k_{-1}) for the dissociation were 0.016 ± 0.020 min^{-1} and 0.022 ± 0.0012 min^{-1}, respectively. The equilibrium dissociation constants of [^{3}H]neuropeptide Y calculated from these kinetic parameters ($K_d = 2.50 \pm 0.075$ nM for SK-N-MC cells and 1.20 ± 0.29 nM for CHO-Y_1 cells) were in good agreement with the corresponding thermodynamic values derived from the saturation binding experiments.

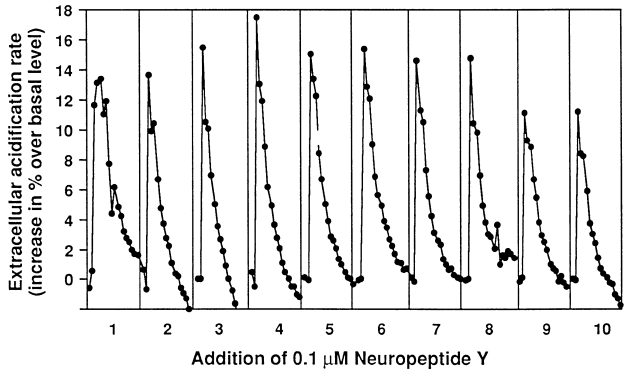

Fig. 8. Repeated challenge of CHO-Y_1 cells with 100 nM neuropeptide Y. The increase in extracellular acidification rate is expressed as % increase above the pre-perfusion level. Each point is the mean of four experiments.

Table 1
The [^{3}H]neuropeptide Y competition binding parameters with BIBP3226, its inactive enantiomer BIBP3435 and neuropeptide Y for SK-N-MC cells and CHO-Y_1 cells

Compound	SK-N-MC cells		CHO-Y_1 cells	
	IC_{50} (nM)	n_H	IC_{50} (nM)	n_H
BIBP3226	17.5 ± 1.7	0.92 ± 0.05	6.49 ± 1.04	0.88 ± 0.03
BIBP3435	2027 ± 845	0.94 ± 0.02	240 ± 31	1.12 ± 0.07
Neuropeptide Y	4.8 ± 1.9	0.99 ± 0.11	0.97 ± 0.23	0.73 ± 0.05

The IC_{50} values were calculated by non-linear regression analysis and n_H represents the corresponding Hill slope. The presented values are the means ± S.E.M. of 3–6 independent experiments.

Unlabelled ligands displaced the binding of [^{3}H]neuropeptide Y to the plated SK-N-MC and CHO-Y_1 cells with the potency order: neuropeptide Y > BIBP3226 ≫ BIBP3435. The displacement was complete; the competition binding parameters are given in Table 1. The Hill slopes of the competition curves were not significantly different from unity, indicating an interaction with a single population of binding sites.

4. Discussion

The neuropeptide YY_1 receptors belong to the G-protein-coupled receptor family and they are known to trigger different cascades of cellular events, including a transient rise in the cytosolic free calcium concentration and a decrease in cAMP production (Wieland et al., 1995; Shigeri and Fujimoto, 1992; Herzog et al., 1992; Gordon et al., 1990). An increasing number of G-protein-coupled receptors have now also been demonstrated to increase the rate at which cells acidify their environment with the degradation products of their energy metabolism. This phenomenon can be conveniently measured by using a microphysiometer and has been claimed to occur independently of the nature of the receptor-induced intracellular events (McConnell et al., 1992). In this study, we show for the first time that this technique can also be used to evaluate the functional consequences of exposure of human neuropeptide YY_1 receptor-containing cell lines to neuropeptide Y and related antagonists. For this purpose, we investigated SK-N-MC cells and CHO-Y_1 cells (i.e., CHO-K1

cells in which the human neuropeptide YY_1 receptor gene has been transfected). As shown in Figs. 1–4, neuropeptide Y was indeed capable of increasing the extracellular acidification rate by up to 15% of the basal level in the CHO-Y_1 cells. This response reached its maximum within 3–4.5 min and then slowly faded away. Under our experimental conditions, the increase in acidification rate did not decline after repeated administration of neuropeptide Y up to about eight times (Fig. 4). This apparent lack of neuropeptide YY_1 receptor desensitization in the microphysiometer assay allowed repetitive challenge of the same cells with neuropeptide Y to obtain dose–response curves as well as antagonist inhibition curves. The neuropeptide Y-mediated increase in acidification rate was clearly related to its ability to stimulate the neuropeptide YY_1 receptors since (a) the novel neuropeptide YY_1 receptor-selective non-peptide antagonist BIBP3226 inhibited this response more potently than its less active enantiomer BIBP3435 did, and (b) neuropeptide Y did not affect the basal acidification rate of the non-transfected CHO-K1 cells. These latter cells do not contain high-affinity sites for [^{3}H]neuropeptide Y and were, therefore, used as a negative control throughout this study.

The effect of neuropeptide Y on the extracellular acidification rate was also investigated in SK-N-MC cells. Although we used exactly the same experimental conditions as for the CHO-Y_1 cells, no change in the acidification rate could be observed for these cells in the presence of neuropeptide Y at concentrations up to 1 μM (Fig. 2). Similarly, the neuropeptide Y receptor agonist peptide YY was also ineffective in these cells (data not shown). However, in agreement with Wieland et al. (1995), our control experiments clearly indicated that neuropeptide YY_1 receptors were present in SK-N-MC cells and that they were functionally coupled with the adenylate cyclase system. Indeed, whole-cell radioligand binding studies with [^{3}H]neuropeptide Y revealed that the density of neuropeptide YY_1 receptors was comparable for SK-N-MC and CHO-Y_1 cells (163 and 188 fmol/mg protein, respectively). For both cell lines, these receptors behaved as an apparently homogeneous class of high-affinity sites for [^{3}H]neuropeptide Y. They also displayed comparable K_d values for [^{3}H]neuropeptide, comparable kinetic constants for association and for dissociation as well as the expected stereoselective inhibition by the antagonists BIBP3226 and BIBP3435. The neuropeptide YY_1 receptors were also functional in SK-N-MC cells since neuropeptide Y produced a dose-dependent and almost complete inhibition of forskolin-stimulated cAMP formation in these cells. The EC_{50} of neuropeptide Y (0.4 nM) for this inhibition was very similar to the K_d for [^{3}H]neuropeptide Y binding. Moreover, as expected for neuropeptide YY_1 receptors, this response was completely and dose-dependently blocked by the neuropeptide YY_1 receptor antagonist BIBP3226, whereas its enantiomer BIBP3435 was much less potent (Fig. 6).

Since both CHO-Y_1 and SK-N-MC cells express neuropeptide YY_1 receptors of human origin, their different ability to increase the extracellular acidification rate upon exposure to neuropeptide Y is clearly unrelated to species-dependent particularities of the receptor structure. The cellular environment could therefore be a determinant factor. Yet, the reasons why some cells do and others do not respond to agonist stimulation with an increase in extracellular acidification are not well understood. In general terms, it is accepted that cell metabolism is only weakly activated by the signalling pathway when G-protein activation is unable to generate a noticeable increase in the extracellular acidification rate. In this context, a number of potential explanations, such as the weak or even absent coupling between the signalling pathway and the Na^+/K^+ ATPase enzyme, have already been put forward (Owicki et al., 1990; Miller et al., 1993). These issues have not been addressed in this study but the observation that neuropeptide Y was able to inhibit cAMP formation in SK-N-MC cells without affecting the acidification rate suggests that the two responses represent independent pathways following receptor activation. At least for G-protein-coupled receptors, this implies that an increased extracellular acidification rate is not merely an obligatory consequence of receptor stimulation. Instead, this phenomenon is more likely to be related to the ability of the receptor to turn on specific and cell-dependent metabolic pathways.

In spite of the similar experimental conditions (37°C, plated cells), the EC_{50} value of neuropeptide Y for increasing the extracellular acidification rate by the CHO-Y_1 cells (38.0 nM) was more than twenty times higher than the K_d value for [^{3}H]neuropeptide Y (1.83 nM) saturation binding. The present observation is not peculiar to the microphysiometer assay or to the use of CHO-Y_1 cells since the reported EC_{50}-value of the neuropeptide Y-induced Ca^{2+} mobilization in SK-N-MC cells (16 nM, Wieland et al., 1995) is also higher than the reported K_d value of [^{3}H]neuropeptide Y binding in this cell line (1.92 nM, this study; 2 nM, Gordon et al., 1990) These findings do not fit with current ideas about the relationship between receptor occupancy and the generation of functional responses, according to which the EC_{50}/K_d ratio should be equal to 1 in the case of a linear receptor occupation–response relationship, or less than 1 when the receptor-mediated response propagates throughout the metabolic network with amplification (i.e., in the case of a 'receptor reserve'). Moreover, it is also noteworthy that the binding of [^{3}H]neuropeptide Y took much longer to reach equilibrium as compared to the time needed for neuropeptide Y to elicit peak responses. Because of the difference in duration between the radioligand binding and the functional assay, the disproportion between the EC_{50}- and K_d-values could be related to non-equilibrium conditions in one of the assays.

Degradation of neuropeptide Y could be held responsible for the high EC_{50}-values in the microphysiometer

assay but this is unlikely since (a) the agonist was continuously refreshed in this type of assay, and (b) the K_d values of the [^{3}H]neuropeptide Y were unaffected when the lengthy radioligand binding assay was performed in the presence or absence of the antiproteolytic agent phenylmethylsulfonyl fluoride (data not shown). Alternatively, it is conceivable that the observed K_d value for the [^{3}H]neuropeptide Y-receptor interaction does not reflect a simple bimolecular process. In this context, several monoamine and peptide receptors are known to adopt a slow agonist-dissociating conformation upon formation of the ternary agonist-receptor–G protein complex. These ternary complexes can be dissociated by GTP and related guanine nucleotides (Birnbaumer and Birnbaumer, 1995), so that the receptors return to their free, low-agonist affinity conformation. In a recent study, it was shown that the addition of guanine nucleotides results in an almost complete lack of [^{3}H]neuropeptide Y binding to its receptors (85% Y_1, 15% Y_2) in rat forebrain membranes (Vanderheyden et al., 1997). This finding suggests that the K_d of [^{3}H]neuropeptide Y is in the nanomolar range for the neuropeptide YY_1 receptor–G protein complex only and, hence, that the radioligand's K_d for the free receptor must be higher.

Tight binding of [^{3}H]neuropeptide Y to a receptor–G protein complex could therefore account for the low K_d value and the slow association and dissociation rate in the present study with CHO-Y_1 and SK-N-MC cells. However, this hypothesis implies that the cell's GTP content becomes depleted after prolonged incubation with the agonist so that the ternary complexes are no longer broken up adequately. Internalisation of the receptor-associated [^{3}H]neuropeptide Y has not been investigated, but it provides an alternative explanation for the binding properties of this radioligand in this study. This phenomenon requires intact cell systems, and for other peptide receptors, such as the endothelin-1 receptor, it has already been held responsible for the almost irreversible binding of [^{125}I]endothelin-1 (Ihara et al., 1995; Wu-Wong et al., 1995a,b). The proposed functional roles of this internalisation process include clearance of the agonist (Wu-Wong et al., 1995a), receptor down-regulation (Benya et al., 1994) and even a prolonged action of the agonist (Resink et al., 1990).

Interestingly, neuropeptide Y did not affect the forskolin-induced cAMP accumulation in CHO-Y_1 cells under exactly the same experimental conditions as those used for the SK-N-MC cells. A similar lack of neuropeptide Y effect on the cAMP levels in these cells was also recently reported by Herzog et al. (1992) and these authors concluded that these cells lack the appropriate $G_{i/o}$ isoform involved in the inhibition of cAMP accumulation. However, this conclusion does not explain the ability of other transfected receptors, such as muscarinic M_2 and M_4 receptors, adenosine A_1 receptors and human cannabinoid receptors, to decrease forskolin-induced cAMP accumulation in CHO cells (Ohnishi et al., 1992; Felder et al., 1993; Akbar et al., 1994; Burford et al., 1995). This discrepancy could, among other causes, be related to the selection of particular cell colonies after transfection and/or to the ability of transfected receptors to affect the adenylate cyclase system in CHO cells in opposite fashions, resulting in attenuation of the prevalent response. This was recently shown for transfected muscarinic M_2 and M_4 receptors (Ohnishi et al., 1992; Burford et al., 1995) and it illustrates the complex relationship between receptors and the adenylate cyclase system in CHO cells.

In conclusion, using the cytosensor, we were able to measure a cell-generated signal (neuropeptide YY_1 receptor-mediated changes in acidification rate) induced by neuropeptide Y in CHO-Y_1 cells in a non-invasive and real-time manner. Our data also suggest that the signalling mechanisms of neuropeptide YY_1 receptors are closely related to the cell type in which they are expressed or, in other words, that the cellular environment determines the type of receptor-induced response.

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