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Desensitisation and internalisation of wildtype and mutant variants of the hY2 receptor

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Title (English)

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Abstract

Wildtype and mutant variants of the Neuropeptide Y receptor Y2 were cloned in HEK293 cells and investigated upon desensitisation and internalisation when stimulated with the ligands NPY, PYY and PYY_{3-36}. It was shown that the wildtype as well as the mutants exhibited low degre of internalisation, and that the internalisation of the wildtype increased linearly, even at saturating concentrations of ligand. A surprising effect suggesting that Adenylate Cyclase may internalise together with the receptor has also been observed.

Keywords

NPY, PYY, PYY_{3-36} ,hY2, GPCRs, Internalisation, Desensitisation, Adenylate Cyclase

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Desensitisation and internalisation of wildtype and mutant variants of the hY2 receptor

Jesper Gantelius

Populärvetenskaplig sammanfattning

Övervikt och fetma är sjukdomstillstånd som väsentligt höjer risken att drabbas av en mängd följdsjukdomar som t.ex. typ 2 diabetes, högt blodtryck, hjärt-kärlsjukdomar samt många former av cancer. I västvärlden är övervikt ett mycket vanligt problem, exempelvis är mer än 50% av de svenska männen och knappt 40 % av kvinnorna överviktiga. Förutom att detta drabbar de enskilda individerna så skapar dessa tillstånd en stor belastning på den allmänna sjukvården och innebär en stor kostnad för samhället.

Aptit styrs i kroppen av ett komplext reglerat system av mekanismer som registrerar energitillgång, samt in och utgående nervbanor till och från viktiga nervcentra. Denna signallering utgörs i synnerhet av en transport av *neuropeptider*, relativt små biologiska molekyler som kan interagera med specifika mottagarmolekyler, *receptorer*, på celler vid nervbanornas slutstation. Dessa celler kan sedan låta signalen gå vidare eller reagera genom att utföra en viss uppgift, till exempel att ändra takten med vilken olika näringsämnen bryts ner eller byggs upp.

När neuropeptider binder till sin receptor under en lång tid och/eller vid höga koncentrationer kan man observera att celler ofta, men inte alltid, startar processer som på olika sätt minskar receptorns förmåga att signallera. Detta kallas *desensitisering*, och sker framförallt genom kemisk modifiering av receptorn, en minskad produktion av nya receptormolekyler samt *internalisering*, vilket innebär att cellen bygger in receptorn i en bubbla av andra molekyler, vilken sedan förflyttas bort från cellytan. Beroende på omständigheterna kan receptorn sedan åter transporteras till cellytan eller brytas ner. Desensitiseringsprocessen är en viktig del av cellers normala funktion och har dessutom stor betydelse för framkallande av beroende och ökad tolerans av t.ex. läkemedel och droger.

I denna studie har vi undersökt desensitiseringsbeteendet hos olika varianter av receptor Y2 som ingår i familjen receptorer till vilka bland annat den mycket vanligt förekommande signalmolekylen Neuropeptid Y binder. Neuropeptid Y, andra relaterade peptider och deras bindning till sina receptorer har visat sig vara viktig för en rad olika fysiologiska processer som t.ex. reglering av aptit, dygnsrytm och blodtryck.

Examensarbete 20p, Molekylär Bioteknikprogrammet

Uppsala Universitet, Maj 2005

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1. Introduction

1.1. Overweight and Obesity

Overweight and obesity are physical conditions assigned to a person having a Body Mass Index (BMI) in the ranges of 25.0-29.9 kg/m^2 (overweight) or 30 kg/m^2 or higher (obesity).

These conditions are coupled to a substantially increased risk of several diseases such as type 2 diabetes, hypertension, stroke, cardiovascular and respiratory issues, gall bladder disease, osetoarthritis, sleep apnoea and endometrial, breast, prostate and colon cancers [1]. In western countries, the percent of the population being overweight or obese according to the above criteria have risen dramatically in the past 20 years, and the World Health Organisation has classified obesity as an epidemic. An estimated 300 000 Americans die every year from obesity-related diseases [2] with approximately 61 % of the US population classified as overweight, including 26 % that also fulfill the criterion for obesity. In Sweden, the fraction of the population classified as obese is clearly lower (fig 1), although the fraction of overweight men surpassed 50 % and the fraction of women is close to 40 %. [3]

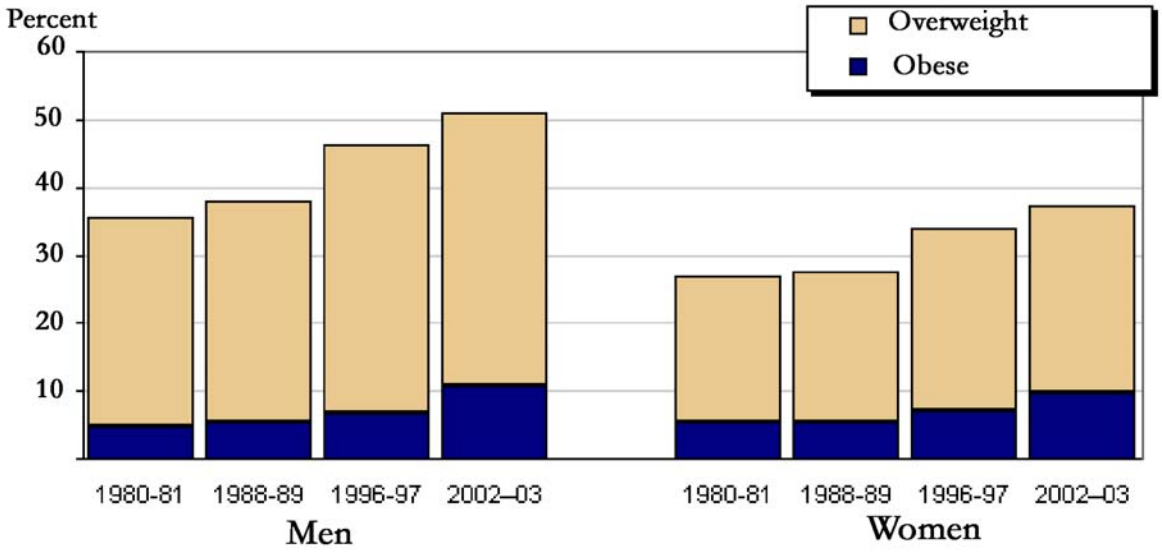

Figure 1. Overview of how the incidence of overweight and obesity have changed in Swedish men and women since the early 80s. The graph was used with permission from Göran Nordström, SCB [3].

The economical strain overweight and obesity produces is substantial, and it has been estimated that the total direct and indirect cost of the conditions on the USA amounted to US$17 billion in the year 2000 [4].

1.1. Energy Homeostasis

The dynamic and rigorously controlled system that matches the body's intake of chemical energy with its energy expenditure is referred to as *Energy Homeostasis*. Piece by piece, the intricate molecular regulatory network that stabilises the energy equilibrium is elucidating itself to scientific scrutiny, and it is now known that several brain centers, such as the hypothalamus, brainstem and reward centra communicate via endocrine neuropeptides to achieve the homeostasis [5]. Hypo- and Hyperphagia as well as pathological deviations from normal metabolism leading to under- or overweight is a complex set of conditions that can arise from an abnormal genetic disposition resulting in suboptimal regulatory networks in and between cells and tissues pertinent to homeostasis, as well as from trauma or environmental or psychological effects.

1.2. Molecular regulation of appetite

The current view is that energy homeostasis is regulated mainly through a number of neuronal circuits that communicate by means of neuropeptides and one can schematically describe the system in terms of sensors, processing units and actuators.

The sensors are cells distributed in tissues that gauge the local energy deficit or surplus. adipose tissue for instance responds to changes in fat-deposits by modulating the level of signalling of, among other, a peptide hormone called Leptin [6]. Leptin levels can then regulate downstream effectors of appetite and energy expenditure. The levels of other endocrine regulators of appetite and energy balance such as Insulin and Adiponectin are also modulated by adipose tissue. The signals stemming from sensoring in the gastrointestinal tract are also of great importance, and some of the hormones released from the gut are the orexigenic factors Ghrelin and the peptides PYY and PP.

The circulating or actively transported hormones released from the energy sensors in fatty tissue and the gastrointestinal tract must then be measured centrally, and decisions must quickly be made in order to keep the feedback system stable. There are several centra for coordinating the primary signals, but of special importance is the Arcuate nucleus (ARC), situated in the Hypothalamus.

The peptides can reach the ARC by means of the median eminence, which is situated below the hypothalamus and lets the peptides bypass the blood-brain barrier (BBB). Some peptides, such as PYY may, however, cross the BBB by non-saturable mechanisms following other paths [7].

Two populations of neurons, one being inhibitory and one being stimulatory, integrate and act upon the inputs from the circulatory peptides [8]. The inhibitory population of neurons reduces food intake by synthesizing the peptide pro-opiomelanocortin (POMC) as well as cocaine- and amphetamine-regulated transcript (CART) [9, 10]. The stimulatory population of cells give rise to a higher level of food intake through the synthesis of neuropeptide Y (NPY) and the agouti-related peptide (AgRP) [11,12].

The resulting stimulatory or inhibitory response produced principally in the ARC is projected further to the Paraventricular Nucleus (PVN) which in turn integrates the incoming levels of NPY, AgRP, melanocortin and other signalling molecules. Being a late step in the signalling process, the levels of the incoming peptides in the PVN are more tightly controlled than the ones in the ARC, resulting in a more sensitive dependence on concentation variations of the input substances. It has for instance been demonstrated that intracerebroventricular injection of NPY into the PVN causes hyperphagia and obesity [13,14].

The PVN will respond to the signalling from the ARC by activating different populations of neurons. The NPY, AGRP and melanocortin projections from the ARC for instance, innervate thyrotropin-releasing hormone neurons in the PVN which can inhibit the gene expression of pro-thyrotropin releasing hormone which has been shown to be a potent modulator of appetite.

1.2.1. PP-fold peptides

A set of important hormones regulating appetite and satiety are the PP (pancreatic polypeptide)-fold peptides, a family that contains primarily Neuropeptide Y (NPY), peptide YY (PYY) and PP, all encompassing 36 amino acids. The PP-fold shows an α-helical domain together with a polyproline helix which is connected by a β-turn. This gives the peptide a U-turn like appearance, and NMR-structures of NPY and PYY can be seen in fig 4.

PP and PYY are circulating hormones that are released postprandially to reflect the number of calories taken in during a meal and are secreted from the endocrine pancreas and the distal gastrointestinal tract, respectively. NPY does not circulate but is expressed in the ARC and reflects an integrated picture of the body's nuitritional status. Its importance can be gauged from the fact that it is the most abundant peptide in the brain, and NPY mRNA levels rise with fasting and drop after feeding [15,16,17]. In addition, when PYY is modified by the enzyme dipeptidyl peptidase IV (DPP-IV) to form PYY_{3-36}, it has been shown to decrease food intake if administrated preprandially to fasted healthy humans, and it has been postulated that PYY_{3-36} binds Y2 receptors on NPY neurones in the ARC, thus inhibiting the appetite stimulation of NPY. Thus, the PP-fold ligands can both stimulate and inhibit appetite, depending on to what populations of cells and receptors they bind.

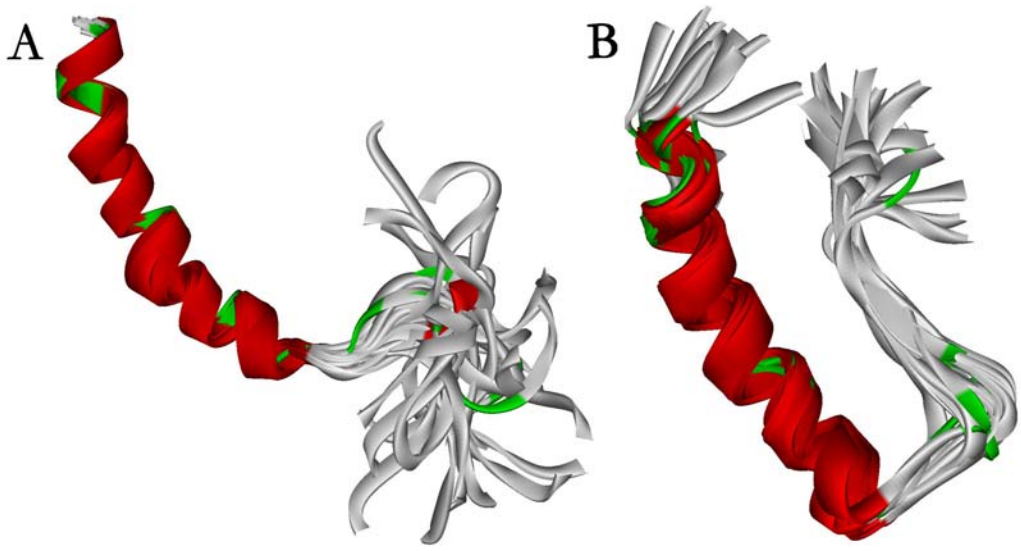

Fig 4. NMR solution-structures of Neuropeptide Y (A, PDB ID: 1RON) and Peptide YY (B, PDB ID: 1RU5). The C-terminus is on the top left for both structures.

1.2.2. Receptors to the PP-fold peptides

The receptors to which the peptides of the PP-fold bind are members of the family of G Protein Couples Receptors (GPCRs) that are characterized by having a structure with a hydrophobic core that spans the cell membrane seven times (hence the synonym 7-transmembrane receptors) with an extracellular N-terminus and a cytoplasmid C-terminus. Also characteristic of the GPCRs is that they signal via the cytoplasmic association and dissociation of G-proteins. A model GPCR can be seen in fig 5.

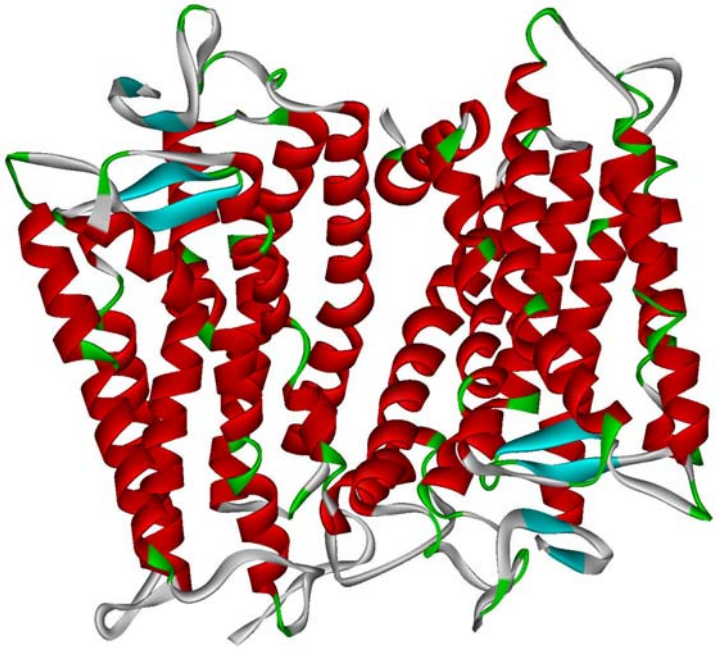

Figure 5. Crystal structure of a model GPCR, Rhodopsin (Dimer). PDB ID: 1F88

It has been suggested that the vertebrate origin of the PP-fold/Receptor system was a single peptide gene and three receptor subtypes, which were the ancestors of today's Y1, Y2 and Y5 [18]. Subsequently, chromosomal duplication events followed by inactivation and divergent evolution has left us with five cloned receptor subtypes in mammals. After the chromosomal duplication events, single gene duplications have produced the peptide variety that we see today. Interestingly, even though the NPY-receptors are clearly subtypes in the same family and bind the same ligands, they have a strikingly low sequence identity, in the range of 27-31%, which is the lowest score seen among any GPCRs that have the same ligand.

The NPY receptors are expressed in various levels in different cells and tissues, and respond differently to ligand stimulation. They also exhibit different affinities to full-length and truncated variants of the PP-fold peptides. In this study, the Y2 receptor has been in focus because of its relevance in appetite regulation and due to its low internalisation behaviour. Y2 is primarily expressed in hippocampus, hypothalamus, amygdala, thalamus and brainstem, and binds truncated fragments of the peptides with high affinity.

1.2.3. Desensitisation and Receptor inactivation mechanisms

It is well known that the respone given when a ligand binds to its receptor in many cases drops if the cell is exposed to large doses of ligand of extended periods of time. This behaviour is called tolerance, and can happen through different mechanisms, principally desensitisation via phosphorylation, internalisation or downregulation.

In the case of GPCRs, the quickest way to desensitise the cell is through phosphorylating the receptor, which is carried out by kinases such as protein kinase A or C (PKA/PKC), or by GPCR kinased (GRKs). Desensitisation by means of phosporylation can be achieved in just a few seconds and it has been demonstrated that phosphorylation of the cytoplasmic parts of GPCRs most often increases the affinity for β-arrestin, which acts both to detach the G-protein from the receptor and by forming a scaffold between the receptor and clathrin proteins. The clathrin proteins can polymerise and mediate endocytosis to remove the receptor and the bound ligand from the surface, transporting the complex to acidic endosomes where the ligand is detached, or to lysosomes where both the receptor and the ligand are degraded [19]. Other mechanisms for internalisation have been demonstrated, although the β-arrestin pathway appears to be the norm. Also pertinent to the present study is that it is known that β-arrestin mediated internalisation can be blocked by 0.5 M Sucrose, presumably through non-specific interactions to the β-arrestin binding sites on the receptor.

The Y1 receptor has been demonstrated to give high levels of internalisation when stimulated, while Y2 has a lower ability to internalise. It seems reasonable that certain domains enhance of inhibit internalisation, possibly by facilitating binding to β-arrestin.

1.2.4. NPY receptor signalling

It has been shown that NPY receptors are associated with $G_{\alpha i}$-proteins, which indicates that their signalling depends on the inhibition of the activity of Adenylate Cyclase. When ligand binds the receptor, $G_{\alpha i}$ dissociates from the heterotrimer G-protein complex and binds to the effector protein Adenylate Cyclase. When this complex is formed, Adenylate Cyclase looses its ability to convert ATP to cyclic AMP (cAMP). Normally cAMP activates Protein Kinase A that in turn catalyses the phosphorylation of other proteins, but upon binding of ligand to a GPCR that signal via G_i, this process is hindered.

There have been nine identified isoforms of Adenylate Cyclase, being differentially expressed in cells and tissues, thus suggesting a complex regulatory mechanism for the cAMP syntheis and consequent downstream signalling.

2. Aims of the project

The potent effects that the PP-fold peptides exert on appetite regulation when they bind to their high-affinity receptors merits them a thorough scientific investigation of the underlying mechanisms for several reasons.

First, one could argue that it is always reasonable to follow a top-down strategy in research effort, focussing first on examining and describing widely occuring and high impact phenomena. The high level ofs of NPY and related peptides in brain tissue and the periphery, and the strong effects that can be seen on administration clearly shows the importance of these systems in normal physiology.

Second, one can easily envisage that the PP-fold peptides or other agonists or antagonists to the receptors could be used therapeutically to stimulate or inhibit appetite in order to treat for instance morbid obesity.

The ability of cells to modulate their response to ligand stimulation is ubiquitous, and one must therefore assume that this is an essential function. One might see the desensitisation behaviour as a means for cells to protect themselves and their downstream signalling partners from ligand stimulation that lies outside the normal signalling range in time or concentration, although it has also been argued that internalisation might be used as a means to dissociate ligands and quickly recycle receptors to the membrane [20]. It is likely that different pathways can be used depending on stimuli although it is unclear how this selective behaviour occurs. Internalisation may however interfere with the effect of paharmaceuticals, especially those that are directed as agonists or antagonists to GPCRs (>50% of all drugs on the market), since a net reduction of receptor numbers on the cell surface will necessarily make the ligand's potency to produce an effect drop.

In this study, the desensitisation and internalisation of the human NPY Y2 receptor has been studied. It has previously been demonstrated that the wildtype Y2 receptor internalises to a lesser degree than the Y1 receptor, but that replacing the C-terminus (CT) of Y2 into Y1 gives almost total internalisation (Lundell unpublished). If, however, the CT-domain as well as the third intracellular loop from Y2 are exchanged into Y1, the internalisation drops from 92 to 34 %, which is the same internalisation level as for wildtype Y1. There is evidence that phosphorylation sites can not only promote internalisation, but in some cases also hinder it [19], and in this case it was hypothesised that the CT of Y2 stimulates internalisation while the third intracellular loop of Y2 hinders it. It was therefore the aim of this study to create and examine desensitisation and internalisation of two Y2 mutants in which two plausable phosphorylation sites in the third intracellular loop were neutralised (Ser244→Ala, Ser251→).

3. Materials and Methods

3.1. Cells

3.1.1. HEK293/EBNA

For binding and internalisation assays a human embryonic kidney cell line, HEK293, that stably expresses the Epstein-Barr nuclear antigen to enhance transfection (hereafter referred to as EBNA cells) has been employed. Cells were grown on 9 cm polystyrene plates (Nunclon^{TM}) in DMEM-F12 (Gibco^{TM}) medium supplemented with 10 % foetal calf serum (FCS, Göteborgs Termometerfabrik), 2.5 µg/ml amphoteracin, 100 units/ml of penicillin, 0.25 mg/ml streptomycin, 0.2 mg/ml hygromycin, 0.25 mg/ml geneticin (all supplied by Invitrogen) and 80 µg/ml tylosine (Sigma) at 37°C, 5 % CO_2. The supplemented medium will hereafter be denoted F12+++, while medium only supplemented with FCS, penicillin and streptomycin will be denoted F12---.

3.2. Cloning of receptors into EBNA cells using the Gateway^{TM} technology

The first step in the Gateway^{TM} procedure is to generate Entry clones, assuming in this text the terminology of the Gateway^{TM} protocol supplied by Life Technologies^{TM}. The Entry clone is characterised as having the desired gene segment flanked on either side by recombination sites called L1 and L2, as well as carrying a Kanamycin resistance gene (Kn^r) for selective growth. The Entry clone can be created by means of standard recombinant DNA methods, and this had previously been done in house to produce the Entry clones used in this project. The clones contained either the ab or cd mutant verions of the hY2 receptor, with either Ser244→Ala (ab) or Ser251→Ala (cd) mutations.

Once an Entry clone has been created and grown in a suitible host, in this case the DH5α competent strain (library efficient) of E.Coli, the clone can be moved into a Destination vector. The Destination vector holds a ccdB gene (translates to a DNA gyrase inhibitor lethal to E.Coli), flanked by R1 and R2 recombination sites. The Destination vector also carries an Ampicillin resistance gene (Amp^r) for selective growth. The Entry clone and the Destination vector are mixed with the LR Clonase Enzyme mix (Life Technologies) in a pool of competent E.Coli, resulting in a recombination event that produces an Expression clone that carries the desired gene, flanked by B1 and B2 sites, as well as an Amp^r gene.

In the recombination event, a small circular by-product is also created which carries the ccdB gene and a Kn^r gene. This methodology ingeniously makes sure that bacteria that have taken up either Entry vectors, Destination vectors, by-product segments, generated nonsense DNA or no DNA at alla will not be able to propagate on Amp-plates due to lack of Ampicillin resistance or because of expression of the lethal ccdB gene. Thus, the only viable colonies are those of the correct expression clones.

In order to guarantee the identity of the expression clones, sequence reactions utilising primers specific to the receptor genes, the entry and destination clones were performed. The resulting DNA was then sequenced on an ABI Prism™ Genetic Analyser and analysed with the Sequences™ software (Gene Codes corp.).

The expression clones were cultured overnight at 37°C in 50 ml Falcon tubes in a LB culture broth followed by plasmid DNA extraction and purification using the Qiagen Midiprep kit.

Plasmid DNA concentrations were assayed on a GeneQuant spectrophotometer (Pharmacia Biotech) and EBNA cells were transfected by simultaneously applying Lipofectamine 2000 transfection agent (Life Technologies) and 12 µg plasmid DNA in Optimem medium to a 9 cm plate of EBNA cells in 90 % confluence (30 µl lipofectamine and 1.5 ml Optimem). The cells were then used for receptor experiments after 2 days of culture.

3.3. Receptor Desensitisation assay

3.3.1. Introducation to the assay

In order to examine the desensitisation of the cells, a commonly employed desensitisation assay was used. As previously described, the NPY receptors signal via the Gα-inhibitory pathway, blocking the effect of Adenylate Cyclase (AC). In this assay, the cells are initially pre-incubated with varying concentrations of ligand. Then, the activity of AC is maximized by a very potent stimulator, Forskolin, after which a short incubation with a high dose of NPY saturates the remaining active receptors. The ability of the cells to inhibit the activity of AC then gives a measure of the level of desensitisation. Basically, if a large number of the receptors have been inactivated or internalised, the saturating dose of NPY won't have any inhibitory effect on AC, and the amount of cAMP produced can thus be used to estimate the desensitisation.

3.3.2. Detailed Procedure

Cells stably expressing the receptor to be analysed were used when having reached confluence. Two 9 cm plates of cells were allowed to metabolise ^{3}H-Adenosine (3 µCi/ml) for three hours in order to have a large fraction of the cell's ATP and ADP exchanged for ^{3}H-ATP/ADP. Cells were then lifted off the plates, spun down for 2 minutes a 500G and resuspended in F12--- media with the desired concentration of agonist for the desired time period and at 37°C. In some experiments, 0.5 M Sucrose was also included in the cell-agonist solution in order to inhibit internalisation.

After the cells had been exposed to the agonist, they were spun down and washed three times in Natrium-Elliot buffer (NaE, 137 mM NaCl, 5 mM KCl, 0.44 M KH_2PO_4, 4.2 mM $NaHCO_3$, 1.2 mM $MgCl_2$*$6H_2O$, 20 mM HEPES, pH 7.4 supplemented with 1 mM $CaCl_2$, 10 mM Glucose and 1 mM IBMX (isomethylbutylxanthine, a phosphodiesterase inhibitor included to block the degradation of produced cAMP)) (first two washes using ice-cold NaE and finally resuspension in 37°C NaE with approximately 1/6 of the total amount of cells suspended in 600 µl suspension), after which they were put on a 96-well plate with the followng configuration: (quadruplicates) First column: 100 µl NaE, 50 µl cells. Second column: 50 µl NaE, 50 µl Forskolin/NaE, 50 µl cells, Third column: 50 µl Forskolin/NaE, 50 µl pNPY (Porcine NPY, Neosystem) in NaE, 50 µl cells. These series of columns were repeated on the plate for all concentrations of ligand used.

The cells were incubated on the plate for 20 minutes at 37°C, after which they were spun down and the pellet was immersed in 200 µl 0.33 M perchloric acid (PCA). After application of the acid, the plate was frozen at -20°C until further analysis.

3.4. Chromatographic separation of cAMP/ATP and calculation of Adenylate Cyclase activation

3.4.1. Chromatographic separation

The samples from the receptor desensitisation assay were processed with a two-step Dowex (BioRad)/Alumina (Sigma) chromatography method [21]. The cell lysates were applied to the Dowex resin together with 750 µl of 0.33 M PCA containing 0.5 nCi/ml of ^{14}C-cAMP for normalisation and controls. After the samples had been put on the column, ATP and ADP were eluted from the Dowex column with 2 ml ddH_2O into scintillation vials and vortexed with 4 ml of Optiphase scintillation coctail (PerkinElmer). Then, cAMP was eluted through elevating the pH gradually by means of adding an additional 10 ml ddH_2O into Alumina columns. Finally, the cAMP was eluted from the Alumina column with 4 ml of 0.1 M Imidazol into scintillation vials and vortexed with 7 ml scintillation coctail. Standard samples were also produced, containing 750 µl 0.33 M PCA and 0.5 nCi/ml ^{14}C-cAMP, 4 ml imidazol and 7 ml scintillation coctail. The scintillation vials were then analysed for β-decays for 5 min/sample in a LKB Wallac 1219 β-scintillation counter.

3.4.2. Estimation of Adenylate Cyclase inhibition

From the Dowex/Alumina separation followed by the monitoring of β-decay, the amount of ATP/ADP as well as cAMP can be estimated. The apparent overflow from ^{14}C to ^{3}H decay is taken into account as well as the efficiency of the columns, estimated as amount of detected eluted ^{14}C divided by the decay from the 750 µl of PCA + ^{14}C-cAMP standard. The inhibition of AC was calculated as 1- ([cAMP]/[ATP+ADP] for NPY+Forskolin divided by [cAMP]/[ATP+ADP] for Forskolin only). The desensitisation could then be estimated through calculating the inhibition at a certain agonist concentration divided by the inhibition produced with no agonist.

3.5. Binding assay for saturation curves and estimation of degree of internalisation

3.5.1. Binding assay

To estimate the degree of internalisation, a radioligand-binding assay was used. Cells were first stimulated with radioligand, after which half of the samples were treated with an acid wash that removes all ligand that is bound to receptors on the surface. All remaining radioactivity must then come from internalised receptor-ligand complexes.

In order to assay the amount of bound ligand to receptors, cells expressing the receptor were incubated on a 96-well plate (triplicates, with two equal sets for acid wash/no acid wash) with relevant concentrations of ^{125}I-PYY (4000 Ci/mmol) in F12--- medium supplemented with 2 g/l bacitracin, either with or without the addition of a 100 nM saturating amount of pNPY. Non-specific binding was defined as the amount of ^{125}I-PYY that could bind in the presence of the saturating concentration of pNPY.

The cells were incubated with the ligand at 37°C for 90 minutes, after which one of the sets was treated with an acid wash step to remove bound ligand (equal volumes cell suspension and 1 M NaCl, 0.4 M NaAc, pH 3) upon which the cells were transferred to a GF/C filter (Wallac) using a TOMTEC 96 harvester (Orange). The filters were pre-soaked in 0.2% polyethyleneimine to reduce specific binding. Following the harvesting, the filters were rinsed with 5 ml 50 mM Tric-HCl, pH 7.4 at 4°C. They were then embedded in Melt-iLex A scintillation sheets (PerkinElmer) after which they were analysed for β-decay on a Wallac 1450 beta counter (PerkinElmer). The data was processed in Prism 4.0 (Graph Pad Software).

3.5.2. Saturation assay

In order to examine how internalisation depends on the ligand concentration, a saturation assay was performed. The total specific binding was measured with or without acid wash for a number of different radioligand concentrations. By inspecting the amount of radioactivity remaining after an acid wash, it was possible to estimate the level of internalisation.

The method is nearly identical to the binding assay, except for making concentration series of the ligand for the cells to incubate in.

3.5.3. Estimation of the degree of internalisation from binding data

The amount of specifically bound ligand was calculated as [total binding] – [non specific binding]. To estimate the degree of internalisation, one calculates [amount of bound ligand (acid wash)]/[amount of bound ligand (no acid wash)].

4. Results

4.1. Generation of hY2-mutants expressed in EBNA cells

The plasmid vectors carrying the ab or cd mutants of the hY2 receptor were previously produced in house. The Gateway protocol was used to generate destination clones with the receptor DNA located in plasmids also containing the Ampicillin resistance gene. Thus, it was possible to select for the right clones through culture on Ampicillin medium. The destination clones were cultured overnight to produce a large batch of plasmid DNA which was then purified through employment of the Qiagen Midiprep kit. To assay that the plasmids contained the correct sequences, some of the purified DNA was used for sequence reactions and analysed on an ABI Prism^{TM} Genetic Analyser.

The purified plasmid DNA was used to transfect EBNA cells which had been grown to 90% confluence through the simultaneous application of Lipofectamine 2000 (Life technologies). The cells were then cultured for two days, after which they were used for binding assays.

4.2. Internalisation of hY2 mutants when exposed to PYY

The EBNA cells expressing the hY2 mutants were harvested upon reaching 90% confluence and incubated with 100 pM of ^{125}I-PYY with or without 100 nM pNPY in a physiological buffer for 90 minutes at 37°C, after which half of the cells were treated with an acid wash for 6 minutes. Then, the cells were transferred to a GF/C filter and analysed for β-decay. It could be seen that the ab and cd mutants had the same or less internalisation as the wildtype, thus indicating that the putative phosphorylation sites targeted did not inhibit internalisation.

Internalisation of hY1, hY2 and mutants

Receptor	% Internalisation	Specific binding	SEM	Specific binding (Acid Wash)	SEM
hY2 wt	7.2	3231	133	551	14
hY2 ab mutant	4.2	1800	100	76	36
hY2 cd mutant	1.4	2403	60	33	31
hY1	20.7	475	76	34	14

Table 1. Internalisation of hY2, hY2 and mutant receptors. SEM=Standard Error Measure

4.3. Saturation and Internalisation of hY2 (wt)

The EBNA cells expressing the hY2 receptors were harvested upon reaching confluence and incubated as duplicates with varying concentrations of ^{125}I-PYY (5-370 pM) in a physiological buffer for 90 minutes at 37°C, after which half of the cells were treated with an acid wash for 10 minutes. Then the cells were washed, transferred to a GF/C filter membrane and assayed for β-decay in a Wallac 1450 beta counter (PerkinElmer). The binding and degree of internalisation can be seen in fig. 6. The results show that the detected radioactivity continues to rise in the samples treated with acid wash, even at the corresponding saturated concentrations. This indicates that the internalisation increases with ligand concentration, showing an almost linear dependence, possibly because of rapid recycling of the internalised receptors back to the cell membrane.

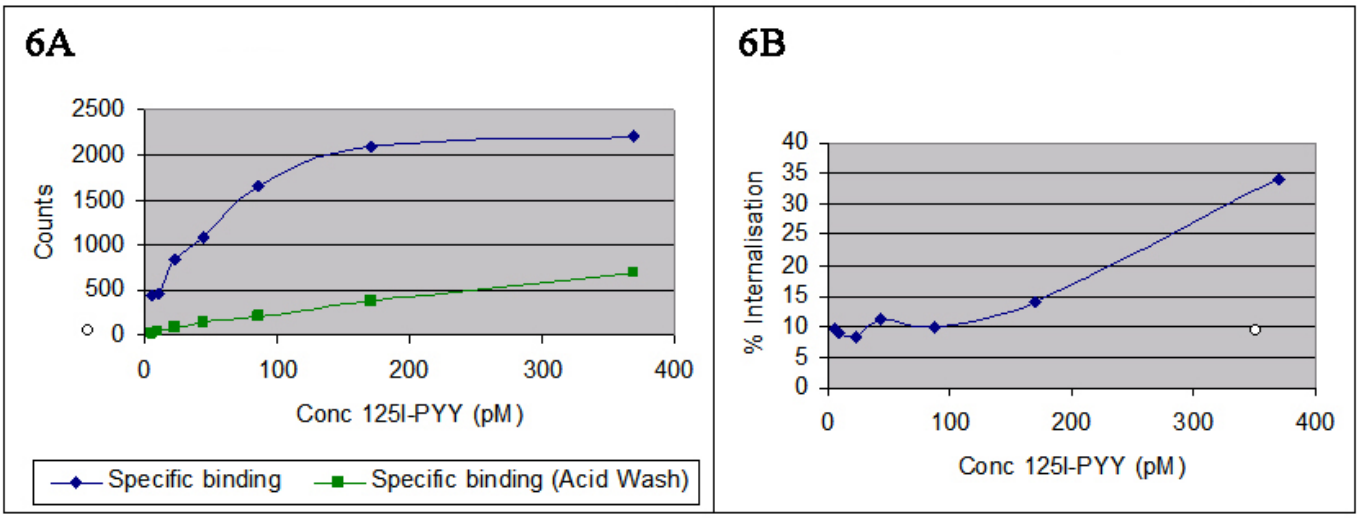

Figure 6: A. Saturation curve for the hY2 receptor with ^{125}I-PYY as agonist.
B. Internalisation behaviour of the hY2 receptor while being exposed to increasing concentrations of ^{125}I-PYY.

4.4. Adenylate Cyclase inhibition assay

The effect of different concentrations of either PYY_{3-36} or pNPY on the ability of wildtype hY2 to inhibit the activity of Adenylate Cyclase was analysed through a widely used cAMP assay. Cells were allowed to metabolise ^{3}H-Adenosine for three hours in order to have a large fraction of the cell's ATP store labelled. The cells were then harvested, washed and incubated for sixty minutes in a physiological buffer containing various concentrations of either PYY_{3-36} or pNPY together with a phosphodiesterase inhibitor (IBMX). To estimate the level of desensitisation reached during this incubation period, the cells were incubated with either the Adenylate Cyclase stimulator Forskolin, Forskolin together with a saturating concentration of pNPY, or just basal medium.

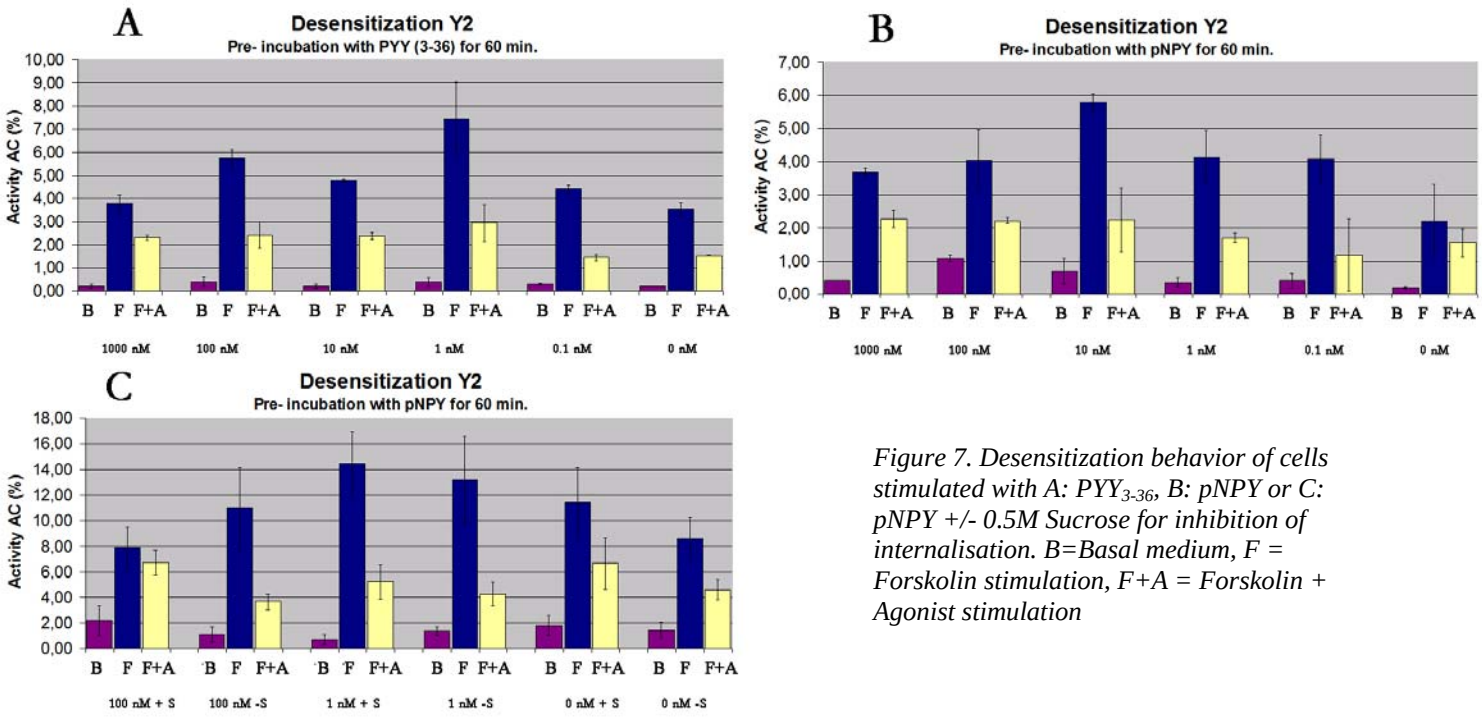

Figure 7. Desensitization behavior of cells stimulated with A: PYY_{3-36}, B: pNPY or C: pNPY +/- 0.5M Sucrose for inhibition of internalisation. B=Basal medium, F = Forskolin stimulation, F+A = Forskolin + Agonist stimulation

5. Discussion

5.1. Internalisation of hY2 variants

A receptor can internalise according to several different pathways, and it is still unclear exactly what componentes need to be involved, and at what time in the process. When trying to find the receptor epitopes that play crucual roles in establishing the receptor's susceptibility to internalisation, it is very reasonable to start to look at sites that usually make a difference, such as putative phosphorylation sites. In this study, it was initially suggested that the low degree of internalisation of the hY2 receptor was due to the presence of one of the two serines in the third intracellular look, and mutant receptors were produced to see if this was the case.

The results did not show any increase, actually a slight decrease, in internalisation for the mutants, which means that the intial hypothesis was wrong, but also that new knowledge was gained.

5.2. Desensitisation of wildtype hY2 receptor

While the results from the cAMP assay consistently displayed high standard deviations and have been hard to reproduce, some general trends can be discerned. It is interesting to note that higher concentrations of NPY do not to a great extent change the level of AC activity when stimulated with Forskolin, but that the Forskolin-induced AC-activity itself drops. This is contrary to the common conception that the forskolin effect on AC is completely independent on the ligand concentration.

However, it has been shown that receptors and their effector molecules often are tightly associated [22]. If this is the case with the Y2 receptor and Adenylate Cyclase, it is possible that a high concentration of signalling ligand gives rise to a large fraction of the receptors being internalised together with AC. If so, the activity of AC will go down accordingly. One can further hypothesise that the internalisation induced by ligand concentration in the range of 10-100nM is lower than the one at 1000 nM, and that desensitisation events dominate. In that case, AC would to a high degree remain associated to the membrane, and could continue to uphold its activity.

5.3. Error sources in methods

5.3.1. Dowex/Alumina

During the timeframe of this project, the viability and level of contamination of the Dowex/Alumina columns have been an issue. Most often, the recovery have been very low and this makes the results questionable. Many different measures in trying to clean and regenerate the columns have been proposed and tried, but in the end it seems like one practically will have to choose between frequently making fresh column material or accepting some hysteresis, or memory effects and leakage of contaminants.

5.3.2. Cloning

Many people have commented on cloning as working like a charm, or not working at all. Sometimes, it is necessary to realise the complexity of the methods that are used, although they may seem like basic routine work. The fact is that biochemical systems are extremely diverse and rules of thumb as well as general methods have to be applied, and because of this there will always be times when the usual approaches don't work, and it is nearly impossible to see why. In this project, the cloning problems were finally solved, although no change in procedure was made.

5.3.3. Peptide levels

When working with chemistry, one will have to ask the question, 'Do I really work with the substances/concentrations that I think I do?'. Many chemical components are unstable and break down spontaneously with time, and in the case of proteins and peptides, adhesion properties are a real issue. It is known that the NPY peptide sticks to plastic surfaces, and the effect may be that the concentration in solution may be 10 % or less than the naive calculation. This makes quantitative analyses impossible to do, unless the sticking effect is either abolished or taken into account. In this project, it is quite possible that adhesive properties of the peptides may have influenced the estimations of the concentrations needed to give internalisation.

6. Future prospects

Desensitisation and internalisation are very important phenomena in pharmacology, and interesting concepts in cell and molecular biology. In the case of the NPY/NPY-receptor system, it would be fruitful to employ other methods to assay the desensitisation and internalisation to verify the achieved results.

In order to resolve the intracellular molecular machinery involved in desensitisation and internalisation, it would also be very valuable to employ protein-protein interaction assays such as immunoblotting, immunoprecipitation, mass-spectrometry or Bioluminescence Resonance Energy Transfer (BRET) to examine what proteins interact with the receptor during signalling, desensitisation and internalisation.

Naturally, it would also be interesting to pursue the investigation of combining different receptor subtypes to find the essential epitopes for desensitisation and internalisation.

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