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Improved Reporter Gene Assays Used to Identify Ligands Acting on Orphan Seven-Transmembrane Receptors

Knut Kotarsky, Niclas E. Nilsson, Björn Olde and Christer Owman

Division of Molecular Neurobiology, Department of Physiological Sciences, Wallenberg Neuroscience Center, S-221 84 Lund, Sweden

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Abstract: Seven-transmembrane G-protein-coupled receptors play a central role in physiology by facilitating cell communication through recognition of a wide range of ligands. Even more important, they represent important drug targets. Unfortunately, for many of these receptors the endogenous ligands, and hence their functions, remain to be identified. These receptors are referred to as “orphan” receptors. A pre-requisite for the identification of ligands activating orphan receptors is powerful assay systems. Until now, reporter gene assays have not been in common use in this process. Here, we summarize our development of improved reporter gene assays. We optimized reporter gene assays in respect of (i) the promoter region of the construct, (ii) the reporter enzyme used, (iii) and the assay procedure. Furthermore, an unique fluorescence-based clone selection step was introduced, allowing rapid selection of the most sensitive reporter cell clones when establishing stable reporter cell lines. Mathematical formulae are provided to enable a simple and reliable comparison between different cell lines, when tested with a compound of interest. The resulting reporter cell lines responded in a very sensitive way to the stimulation of various test receptors. The reporter system was termed HighTRACE® (high-throughput reporter assay with clone election). Its high assay quality makes it suitable as a primary screening tool. Ligands for two recently unknown 7TM receptors were identified using the HighTRACE® system i.e., two cell surface free fatty acid receptors, GPR40 (FFA1R) and GPR43 (FFA2R). The identification was accomplished using a reverse pharmacology approach.

The seven-transmembrane (7TM), G-protein-coupled receptors comprise the largest family of cell surface receptors. A wide variety of molecules interact with and activate these receptors. The list of such ligands includes signaling molecules functioning as hormones, neurotransmitters, chemoattractants, or molecules formed outside the body, such as odorants, pheromones, nutrients, and even light. The ligands display widely different sizes (from larger proteins to small molecules) and belong to different chemical classes (lipids, amino acids, ions, or proteins). Despite these differences, the receptors show a common general architecture, including seven-transmembrane helices (recently reviewed by Lefkowitz 2000; Pierce et al. 2002; Schoneberg et al. 2002). It is generally assumed that they also function in a similar way, even when the ligand binding domains, or pockets, are not totally conserved. The only three-dimensional structure of a 7TM receptor known today from studies of its crystal structure is that of bovine rhodopsin. The structure was determined by X-ray crystallography; however, it shows the receptor in its inactive conformation (Palczewski et al. 2000). Attempts have been made to superimpose other 7TM receptors onto the rhodopsin model in silico (Paterlini 2002). Differences in the primary sequences and in the ligand binding make receptor modeling difficult (Archer et al. 2003). Hence, the identification of ligands acting on 7TM receptors cannot be predicted from structural features but is still completely based on experimental methods, involving mass screening and other high throughput approaches.

Orphan 7TM receptors

Seven-transmembrane, G-protein-coupled receptors play a central role in physiology since they facilitate cell communication in multicellular organisms by recognition of a broad range of ligands. They also represent important drug targets. Unfortunately, for many of these receptors the endogenous ligand(s) and, hence, their functions, remain to be identified. These receptors are referred to as “orphan” receptors.

The identification and characterization of such orphan, 7TM receptors have attracted much interest over the last decade. This focus on orphan 7TM receptors can be explained by two facts. Understanding cell communication, and eventually physiological mechanisms, requires both knowledge about the particular signaling mediators and
their receptors. An even more important fact is that some fifty percent of all drugs in clinical use interact with 7TM receptors, making this family of receptors an important target for the development of drug candidates and the evaluation of lead compounds (Wilson et al. 1998). Hence, attempts to identify orphan 7TM receptors have not been driven by a purely academic interest, but have rather been carried out in a situation characterized by competition between academy and industry (Fujii 2000; Hosoya et al. 2000; Howard et al. 2000; Kojima et al. 2000; Raddatz et al. 2000; Shan et al. 2000; Szekeres et al. 2000; Funes et al. 2002).

Estimations of the number of orphan 7TM receptors that vary between one hundred and several hundreds (Civelli et al. 2001; Howard et al. 2001; Im 2002; Szekeres 2002; Vassilatis et al. 2003). Such estimations usually do not include the odorant receptors. The total number of 7TM receptors has been estimated to 3–4% of all genes in humans and 5% in Caenorhabditis elegans (Bockart & Pin 1999; Bargmann 1998).

Requirements on primary assay systems

In attempts to identify ligands acting on 7TM receptors the receptor protein is used as a “fishing tool”, either in binding assays or in functional tests. A plethora of different assay systems has been described in the literature. However, due to the tough requirements placed on the assay to be used in the initial characterization, systems actually applied for identification purposes are less numerous. Assays have to display a high reliability and quality, a property that is defined by a high Z-factor. The Z-factor combines the difference between background and signal, with the differences in the standard deviation of both values in a number between 0 and 1, where 1 stands for the theoretically ideal assay (Zhang et al. 1999). A Z-factor greater than 0.5 is required for an assay used as a primary screening tool (Zhang et al. 1999; Johnston 2002).

Assay systems used as a primary screening tool, apart from reporter gene assays (Hill et al. 2001; Kotarsky et al. 2003a), include methods to detect changes in the intracellular Ca2+ concentration (Tanahashi et al. 1990; Button & Brownstein 1993; Tryselius et al. 2000; Kotani et al. 2001; Niedernberg et al. 2003), binding assays (Wang et al. 2001), as well as assays based on yeast cells (Chambers et al. 2000; Brown et al. 2003) and on Xenopus oocytes (Bachner et al. 1999; Heise et al. 2000). The techniques used as primary screening tools have been reviewed recently (Hill et al. 2001; Szekeres 2002).

Reporter gene assays

A widely used screening approach is the reporter gene technology (for review see Hill et al. (2001) and Naylor (1999)) It requires a DNA-based reporter construct, which usually consists of a promoter and a reporter gene, that are stably integrated into the genome of the cell. The transcriptional activity of the reporter gene is eventually regulated via the intracellular signal transduction mechanisms by the receptor present on the cell membrane. The receptor can be either endogenously expressed or in a recombinant form.

Early reporter constructs contained whole gene promoters, which were regulated upon various stimuli (Voraberger et al. 1991; Weyer et al. 1993; Stratowa et al. 1995). An increasing knowledge about, e.g., transcription factor binding sites and signaling pathways lead to the use of reporter constructs containing one or several copies of a response motif in their promoters (Sista et al. 1994; Chen et al. 1995; Boss et al. 1996). Certain transcription factors that are activated in response to the stimulation of different signaling pathways by the different G-proteins (table 1) can interact with specific response motifs. For example, receptors coupling to Gsα activation have usually been monitored with reporter constructs containing cAMP-responsive elements (CRE) (Chen et al. 1995). Activation of Goq and Gsα-coupled receptors has been performed using promoters which contain either TPA-response elements (TRE) (Sista et al. 1994, Kotarsky et al. 2001), serum response elements (Lin et al. 2002), nuclear factor of activated T-cells (NFAT) response elements (Zlokarnik et al. 1998; Kunapuli et al. 2003), or nuclear factor κB (NF-κB) response elements (Moon et al. 2001; Kotarsky et al. 2003a).

Advantages and limitations of reporter gene assays.

Reporter gene assays have been used in numerous pharmacological studies investigating full or partial agonists, antagonists or inverse agonists (Hill et al. 2001; Johnston 2002). They are well suited to identify second messenger pathways and signaling cascades down-stream from the receptor to the nucleus (Gonzalez & Negulescu 1998). They have been applied to the process of “de-orphanization”, as primary or secondary screening tools (Briscoe et al. 2003; Kawamata et al. 2003). Reporter gene assays are well suited for high-throughput screening approaches in various assay formats (Hill et al. 2001).

However, the application of reporter gene assays is limited by certain features inherent to the assay itself. In order to establish a reporter gene assay, cells need to be stably transfected with several constructs. Stable integration of the reporter construct is a prerequisite for an efficient clonal selection (Kotarsky et al. 2001 & 2003a). In order for the test cells to produce the reporter enzyme an incubation time at 37°C of several hours is unavoidable. A potential interaction with other steps in the signaling cascade may be possible under that time and has to be excluded by appropriate controls. The signal amplification in reporter gene assays may change signaling behaviour of low-efficacy agonists, which may instead appear as full agonists. Even here appropriate controls, as an internal standard (Kotarsky et al. 2003b) that fully activates the reporter will be of great value.

Optimization of the promoter region.

Our interest in 7TM receptors, displaying a high degree of sequence similarity to receptors for inflammatory mediators in
Some transcription factors and their response motifs activated by 7TM receptors.

<table>
<thead>
<tr>
<th>Transcription factor</th>
<th>Response element</th>
<th>Activated by</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CREB family</td>
<td>CRE</td>
<td>Goα</td>
<td>(Chen et al. 1995)</td>
</tr>
<tr>
<td>AP-1 (Fos and Jun)</td>
<td>TRE</td>
<td>Goα, Goβi</td>
<td>(Sista et al. 1994)</td>
</tr>
<tr>
<td>Serum response factor</td>
<td>SRE</td>
<td>Goα12, Goα13, Goαq/11</td>
<td>(Lin et al. 2002; Suzuki et al. 2003)</td>
</tr>
<tr>
<td>NF-κB</td>
<td>NF-κB</td>
<td>All except Goα</td>
<td>(Ye 2001)</td>
</tr>
<tr>
<td>STAT</td>
<td>STAT</td>
<td>Goαq</td>
<td>(Pelletier et al. 2003)</td>
</tr>
<tr>
<td>NFAT</td>
<td>NFAT</td>
<td>Goαq</td>
<td>(Boss et al. 1996)</td>
</tr>
</tbody>
</table>

Question mark indicates that G-protein involvement is controversial.

The use of consensus response elements in the promoters of reporter gene constructs, rather than complete promoters of genes, had been introduced earlier (Chen et al. 1995; Boss et al. 1996). Czernilofsky et al. (1998) tested constructs containing 3 and 6 TRE transfected into COS cells, and recognized an increase in signal amplification in the latter constructs.

The promoter region was further optimized in an approach to expand the applicability of the reporter vector described (Kotarsky et al. 2003a). Multiple copies of a newly designed response motif were included into the reporter construct, which contained the consensus motifs of NF-κB and STAT binding sites. The reporter vector was also equipped with the minimal FOS promoter; it was named pcFUS3 (fig. 2) and was used for the construction of HeLa HFF11 cells. Reporter constructs containing two different kinds of transcription factor consensus motifs in their promoters have also been accomplished by Fitzgerald et al. (1999) by combining three multiple response element with a CAMP response element.

**Choice of the reporter enzyme.**

A useful reporter enzyme should display little or no background in eukaryotic cells and, in order to facilitate clone selection, it should exhibit a single cell resolution. Luciferases, which are often used as reporters, do not have any internal background in mammalian systems and are, hence, very useful in plate-reader formats. However, luciferase activity is usually analyzed in lysed cells. Detection at a single cell level is not convincing and requires substrate loading of the cells (Craig et al. 1991; Greer & Szalay 2002).

In order to analyze reporter gene activity at a single cell level, β-galactosidase and β-lactamase have been used (Fiering et al. 1991; Zlokarnik et al. 1998; Knapp et al. 2003). Both require loading cells with substrate molecules, which are then converted into fluorescent products through the activity of the reporter enzyme. Besides this, cells have an intrinsic fluorescent background which makes these techniques less sensitive when used in a plate reader. In case of the β-lactamase assay, this problem has been addressed by using the ratio of two fluorescence wavelengths, rather than absolute changes in the fluorescence signal (Kunapuli et al. 2003; Zlokarnik et al. 1998).
Green-fluorescent protein (GFP), on the other hand, displays an intrinsic fluorescent property due to its three-dimensional structure. This builds up a fluorophore consisting of three amino acids in tight proximity to each other (Prashar et al. 1992; Cody et al. 1993). A variety of GFP variants with different absorption and emission spectra have been described (Barak et al. 1997; Miyawaki et al. 1997; Kain 1999; Lenkei et al. 2000; Nagai et al. 2001). GFP is easily detected at a single cell level using fluorescence microscopy or flow cytometry, and it has also been used in plate-reader assays (Barak et al. 1997; Kain 1999). However, due to the intrinsic fluorescence of the cells the signal windows are rather small (Ghose et al. 1999).

In order to combine the advantageous properties of firefly luciferase and GFP, a chimeric protein based on a GFP-luciferase fusion gene was constructed (Kotarsky et al. 2001). The GFP gene was placed upstream of the firefly luciferase gene. When expressed in mammalian cells both partners retained their enzymatic properties. Reporter enzyme activity in transfected cells could easily be detected using either fluorescence microscopy, flow cytometry, or plate readers by a very high signal-to-background ratio and high Z-factors (Kotarsky et al. 2001 & 2003a).

Clone selection of reporter cell lines.
The single cell resolution displayed by the reporter enzyme has enabled an efficient clone selection procedure, which is either performed by fluorescence activated cell sorting (FACS) or by fluorescence microscopy. Instead of testing a large number of clones selected at random, cell clones resulting from a stable transfection were stimulated with a protein kinase C (PKC) activator, and cell clones showing a high EGFP (enhanced green-fluorescent protein) expression were subsequently picked, expanded, and retested (fig. 1). This clone selection step greatly simplified the identification of sensitive reporter cell clones. Whereas 90% to 95% of all cell clones selected with this method expressed firefly luciferase, cell clones selected in the conventional manner often failed to either express EGFP (85%), luciferase (72%), or both (65%). Using the present approach it was sufficient to test 20–30 cell clones that expressed the highest level of EGFP after PMA stimulation in order to establish a well-responding reporter cell line. The best responding clone after three to four subsequent testings was chosen. The described reporter gene assays, including the described efficient clone selection, has been named HighTRACE® (high-throughput reporter assay with clone election) (Kotarsky et al. 2001).

Stable cell lines were established by the clone selection procedure described above. The cloning gave raise to either HF1 cell line (using pcFUS2), or the HFF11 cell line (using pcFUS3).

Example of 7TM receptors expressed in HighTRACE® reporter cell lines.
In further experiments the ability of several receptors to increase reporter enzyme activity was investigated after stimulation with the respective agonist. For this purpose the first leukotriene B4 receptor (BLT1), the fifth CC chemokine receptor (CCR5), and the rat α1b receptor were transfected into the HF1 reporter cell lines. These gave rise to the HF1pBLT1, HF1pCCR5, and HF1pRα1b reporter cell lines.

The reporter cell lines were subsequently used in ligand stimulation experiments in order to obtain EC50 values from the concentration-response curves. All tested reporter cell lines responded well to full agonist stimulation (CCR5/ RANTES 15 times, rat α1b /epinephrine 50 times, and BLT1/LTB4 200 times), whereas control cells did not respond. The obtained EC50 values were in good agreement with previously published results for these receptors (Yokomizo et al. 1997; Boie et al. 1999).

In contrast, other described reporter gene assays employ polyclonal cells or, more often, they test a small number of clones in a rather laborious process (Weyer et al. 1993; Sista et al. 1994; Goetz et al. 2000). The resulting reporter cell lines increased reporter protein activity at maximum stimulation by 3 to 20 times when stimulated with a strong PKC activator (Weyer et al. 1993; Sista et al. 1994).

In further experiments both cell lines, HF1 (containing pcFUS2) and HFF11 (containing pcFUS3) (fig. 2), were used in comparative studies. Experiments were performed using three different 7TM receptors as examples. The receptors were either recombinantly (complement C5a) or endogenously (CXCR4 and ATP-receptors) expressed in both cell lines. In all three cases it was found that the HFF11-based cell lines increased luciferase expression stronger in response to stimulation than the HF1-based cell lines (Kotarsky et al. 2003a).

Other receptors functionally expressed in HFF11 cells include the following 7TM receptors: BLT2R, CysLT2R, C3aR, PAR1 and the fatty acid receptors previously named GPR40, GPR41 and GPR43 (see below). Notably the design of the reporter enabled us to monitor also the activation of tyrosine kinase coupled receptors (e.g. EGF receptor).

Fig. 2. The design of the reporter constructs used to establish the stable cell lines HF1 (pcFUS2), HFF11 (pcFUS3), and HR36 (pcFUS3.sRL).
Assay systems based on secreted reporter enzymes.

A further type of reporter construct was designed in order to simplify the assay procedure. Whereas firefly luciferase and many other reporter enzymes used in 96-well plate assays are based on their intracellular expression followed by analysis in cell lysates, other reporter enzymes, such as secreted alkaline phosphatase (Yang et al. 1997; Moon et al. 2001), or secreted luciferases (Thompson et al. 1990; Liu et al. 1997; Liu & Escher 1999; Tanahashi et al. 2001; Greer & Szalay 2002), can be analyzed in the supernatant of the cells. This simplifies the assay procedure in two ways: (i) it is not necessary to lyse the cells before analysis, which eliminates several steps in the cell handling, and (ii) normalization can be performed for each individual well before and after ligand stimulation (fig. 3). This will also improve assay quality.

While secreted alkaline phosphatase requires a heat-inactivation step in order to reduce the endogenous background, the luciferases can be analyzed immediately by adding their respective luciferins. The luciferin of the decapode _Vargula_ is difficult to obtain commercially at reasonable costs; however, coelenterazine which may be used as luciferin for the _Renilla_ luciferase provides an alternative. The _Gaussia_ luciferase, which also uses coelenterazine as substrate, has recently been cloned and may offer a higher quantum yield than _Renilla_ luciferase (Verhaegent & Christophoulus 2002).

Against this background, a reporter construct was designed containing cDNA encoding two reporter enzymes: a secreted, genetically modified _Renilla_ luciferase (Liu et al. 1997; Liu & Escher 1999), and a second intracellular reporter, composed of EGFP and firefly luciferase (fig 2). The expression of both enzymes was coupled using a short synthetic internal ribosomal entry site (IRES) (Edelman et al. 2000; Owens et al. 2001).

_Renilla_ luciferase enzyme activity was easily detected in the cell supernatant. However, the expression of EGFP could not be detected using fluorescence microscopy or flow cytometry, not even in PMA-stimulated reporter cells. The cell lysate displayed small, though detectable, amounts of firefly luciferase activity using a plate luminometer. This indicates that the IRES directed the translation machinery to the second, intracellular reporter enzyme in a rather inefficient way. The amounts of intracellular reporter produced per cell were too small to allow for an efficient clone selection based on EGFP fluorescence.

Among 36 clones tested in an old-fashioned clone selection procedure, a clone designated HR36 was chosen for comparison of two different assay procedures. In the first approach, cells grown in 96-well plates were exposed to different ATP concentrations to stimulate endogenously expressed ATP receptors. In a second approach, cells grown in batch culture were suspended in an EDTA solution and added to the ligands present in a 96-well plate. _Renilla_ luciferase activity was analyzed in the supernatant. The second approach simplifies assay performance, as it involves fewer steps in cell handling and at the same time it increases assay quality (fig. 3).

Strategies to identify orphan 7TM receptors

Generally, two different strategies have been used for the identification of orphan receptors. The pharmaceutical industry and some academic laboratories often use an approach known as reverse pharmacology. This implies the use of huge substance libraries, which are matched with recombinant orphan receptor libraries in attempts to find positive hits. Smaller laboratories, on the other hand, use a targeted approach often on “strange” receptors, over many years in laborious procedures to identify the ligand for a specific receptor. The second approach usually starts from a tissue extract as the source of the unknown ligand, and is often referred to as the orphan receptor strategy (Civelli et al. 1997, 1998, 1999 & 2001).

While the reverse pharmacology approach is mainly limited by the design of the substance and receptor libraries, the second approach relies completely on the presence of the ligand in a tissue extract. Ligand stability and low concentration of the ligand in a tissue extract of limited accessibility may further complicate ligand purification and subsequently identification.

To overcome these problems indirect approaches have been applied. Receptors with a high degree of sequence similarity are usually activated by similar ligands. Even the tissue distribution of the receptor may give certain clues.
about the nature and function of the ligand. That in combination with the knock-out of the receptor in an animal model may provide information about physiological and pathophysiological processes the receptor is involved in.

Application of HighTRACE® to the identification of fatty acid receptors

Using a reverse pharmacology approach we tested ten reporter cell lines, each expressing a different recombinant orphan 7TM receptor, in a screen aimed to identify possible fatty acid receptors. For this approach HFF11 reporter cell lines (Kotarsky et al. 2003a) were used. A schematic drawing is depicted in fig. 4.

A mock-transfected control cell line and reporter cell lines each expressing a different orphan 7TM receptor were seeded into multi-well dishes and were stimulated with test substances (TSi,n), the negative control (solvent), or the positive control (10^{-6}M PMA). For each single well tested with a TS light production was normalized using equation 1 in fig. 4. After calculating the average μ and standard deviation of the respective samples, the values derived from the TS tested on a cell line expressing a given receptor were compared with the values originated from a test cell line, which was mock-transfected and did not express any orphan 7TM receptor (fig. 4, equation 2). Equation 2 is used to calculate the Z-factor according to Zhang et al. (1999). An expanded use of the original formulae had been earlier suggested (Zhang et al. 1999). A Z-factor between 0.5 and 1 is strong evidence that TSi has an effect on the cell line expressing receptor M; whereas a Z-factor below 0 will occur in the absence of a stimulating interaction between the TS and the orphan receptor. The here described approach might be used to compare multiple cell lines, increasing the reliability of the method.

In our experiment (Kotarsky et al. 2003b) the cell line expressing the orphan receptor, GPR40 (Sawzdargo et al. 1997), showed a significant increase in luciferase activity when stimulated with 50 μM linoleic acid, with a Z-factor of 0.8.

Numerous fatty acids and their derivatives were subsequently tested using the GPR40-expressing HFF11 reporter cell line. Fatty acids with medium to long chain length and with different degree of saturation or hydroxylation all gave an increase in luciferase activity (table 2). However, short-chain fatty acids (C1-C4) had no effect on cells expressing GPR40 (up to 1 mM tested). Because of the activation of GPR40 by a wide range of fatty acids, along with its tissue distribution and its proposed physiological role, we suggested the name “free fatty acid receptor”, FFA1R, for this receptor.

The identification of GPR40 (FFA1R) as a receptor for medium-to-long chain free fatty acids was independently described by two other groups (Briscoe et al. 2003; Itoh et al. 2003), corroborating our main findings (table 2). Differences in the reported EC50 values most probably reflect the different assay systems used for the identification. However, FFA1R seems to play an important physiological role since it modulates insulin secretion from pancreatic β-cells upon stimulation with free fatty acids (Itoh et al. 2003) and also responds to modern antidiabetic drugs (Kotarsky et al. 2003b) of the thiazolidinedione type (Cantello et al. 1994; Reginato et al. 1998).

A second orphan receptor, GPR43, activated by short-chain free fatty acids, was identified using the techniques described above (Nilsson et al. 2003). This receptor, mainly expressed on peripheral blood leukocytes (Nilsson et al. 2003; Senga et al. 2003), was most potently activated by acetate and propionate. Its existence may explain various effects of these short-chain fatty acids on immune cells under normal and pathophysiological conditions (Brown et al. 2003; Le Poul et al. 2003; Nilsson et al. 2003).
Conclusions

The identification of natural and surrogate ligands acting on 7TM receptors involve mainly cell-based assay systems. Hitherto, reporter gene assays have not been used extensively in this process. Oda et al. (2000) and Zhu et al. (2001) applied reporter gene techniques to the identification of a fourth histamine receptor (Oda et al. 2000; Zhu et al. 2001), and Kawamata et al. used a reporter gene assay to identify the first 7TM receptor responding to bile acids. In all these instances reporter gene assays were applied as primary screening tools, although they have also been used as secondary assays (An et al. 1997; Briscoe et al. 2003).

The design of improved reporter gene assays can be accomplished by optimizing the promoter region of the construct, the reporter enzyme used, and the assay procedure. Further, an efficient clone selection step allowing the election of the most sensitive reporter cell clones will increase the signal-to-background ratio markedly. As shown above in some examples, the test cell lines we have established responded very efficiently to stimulation of various cell surface receptors (Kotarsky et al. 2001 & 2003a). The amplification of the reporter enzyme activity was substantially larger than in any earlier described system, and its high assay quality makes it suitable as a primary screening tool. Reporter gene assays comprising an improved signal-to-background ratio and a high Z-factor have turned out to be useful for the identification of ligands acting on orphan 7TM receptors in a primary screening approach.

As presently exemplified, this led us to the discovery of the first and second cell surface, free fatty acid receptors, FFA₁R and FFA₂R (Kotarsky et al. 2003b; Nilsson et al. 2003). The identification of these two novel receptors provides an explanation for many physiological effects attributed to free fatty acids, that have hitherto remained unexplained and which previously have mainly been seen as energy sources and cell constituents. The activation of FFA₁R by thiazolidinedione-type anti-diabetic drugs implies an important connection to type II diabetes, which is confirmed by the action of FFA₁R on insulin secretion from pancreatic β-cells (Itoh et al. 2003). The receptor discovery will help to increase our understanding of the underlying mechanisms of this and other life-style diseases.

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