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Detecting ligand interactions with G protein-coupled receptors in real-time on living cells

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ABSTRACT

G protein-coupled receptors (GPCRs) are a large group of receptors of great biological and clinical relevance. Despite this, the tools for a detailed analysis of ligand–GPCR interactions are limited. The aim of this paper was to demonstrate how ligand binding to GPCRs can be followed in real-time on living cells. This was conducted using two model systems, the radiolabeled porcine peptide YY (pPYY) interacting with transfected human Y2 receptor (hY2R) and the bombesin antagonist RM26 binding to the naturally expressed gastrin-releasing peptide receptor (GRPR). By following the interaction over time, the affinity and kinetic properties such as association and dissociation rate were obtained. Additionally, data were analyzed using the Interaction Map method, which can evaluate a real-time binding curve and present the number of parallel interactions contributing to the curve. It was found that pPYY binds very slowly with an estimated time to equilibrium of approximately 12 h. This may be problematic in standard end-point assays where equilibrium is required. The RM26 binding showed signs of heterogeneity, observed as two parallel interactions with unique kinetic properties. In conclusion, measuring binding in real-time using living cells opens up for a better understanding of ligand interactions with GPCRs.

1. Introduction

G-protein coupled receptors (GPCRs) constitute a large class of receptors of great biological importance. Abnormal expression, regulation and function have been linked to many diseases, making them important targets in diagnostic and therapy. The receptors transduce extracellular signals through the membrane via a number of conformational changes, but despite clinical relevance the knowledge about their molecular mechanisms is limited [1,2].

There are several methods to identify compounds that target GPCRs. Ligand-receptor binding assays, such as saturation assays, are widely used methods to determine the ligand-receptor affinity. Other strategies include indirect signaling assays, where binding to GPCRs is instead monitored by following the activity of the downstream signaling pathway. Although much information can be gained from such assays, a general drawback is that the downstream effect will depend on other components present in the cell, making it difficult to generalize results [3,4]. Furthermore, both saturation assays and activity assays are most often end-point measurements, which typically rely on equilibrium being reached during the time of incubation. This has been identified as a potential source of error, since high affinity binders may have equilibration times of many hours [5,6] and protocols with a few hours of incubation are still common.

An alternative to the assays described above is to monitor the ligand-receptor binding in real-time. These measurements do not depend on equilibrium and further provides an inherent quality control, where assay problems can immediately be detected as irregularities in the binding curve. More importantly, time-resolved interaction measurements provide information about not only the affinity, but also the binding kinetics, i.e. the association and dissociation rates. This is particularly important during drug development, where it is crucial to know if the drug will have enough time to bind the target before it is cleared from the blood, and where the biological effect depends on residence time, i.e. how long a drug remains bound to its target [7]. Because of the

Abbreviations: GPCR, G protein-coupled receptor; pPYY, porcine peptide YY; hY2R, human Y2 receptor; GRPR, gastrin-releasing peptide receptor; ^{125}I, iodine-125; ^{111}In, indium-111.

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information-rich nature of real-time interaction data, new tools have emerged to extract additional information from such data. One example is the analytical procedure Interaction Map, which can decipher the degree of interaction heterogeneity from time resolved binding traces, to understand if a molecule binds to its target in different manners [8]. Such heterogeneity can be caused by various conformations, molecular variants or dimer states and will affect the interaction kinetics, visible through Interaction Map.

So far most real-time interaction studies have been performed in cell free systems, using e.g. the sensitive and label-free technology of surface plasmon resonance (SPR) [9,10]. However, these measurements are rarely straightforward due to difficulties in overexpression, purification and stabilization of the GPCRs [10,11]. There may further be discrepancies between the SPR results and effects in living systems, due to the lack of G-protein and stabilizing membrane components. Similar observations have been made in other systems, where large differences between SPR data and cell data were observed [12].

The aim of the present study was to establish a method for real-time measurements of interactions with GPCRs in living cells. This was conducted using the instrument LigandTracer®, which has been described in detail and validated previously for overexpressed receptors in cancer cells [13,14]. In brief, adherent cells expressing the target protein are seeded in a local part of a circular Petri dish, which is placed on an inclined, rotating support with a detector positioned over the elevated part of the dish (Fig. 1A). A buffer containing a radiolabeled molecule, e.g. a peptide or protein, is added. If the molecule binds to the target on the cells, the detector will register a peak each time the cell area passes by the detector, using a cell-free area of the dish as a reference. By following the peak height over time, a real-time binding trace is obtained. The method was originally developed for a single cell area, but after further development at least three areas can be studied in parallel, enabling e.g. comparison of binding to different cell lines (Fig. 1B).

The use of living cells in time-resolved binding assays has the potential to improve understanding of GPCRs in their true environment. Two model systems were used to evaluate the method and demonstrate proof-of-concept. In the first study, the binding of the pPYY peptide to cells expressing different degrees of the human neuropeptide receptor hY2R [15] was measured. In the second study, the interaction between the bombesin antagonist RM26 developed for medicinal imaging of cancer and the gastrin-releasing peptide receptor GRPR [16] was detected. The binding showed clear signs of interaction heterogeneity and was therefore further evaluated using Interaction Map.

2. Materials and methods

2.1. Reagents and labeling

The human peptide YY pre-labeled with ^{125}I (denoted ^{125}I-pPYY) was purchased from PerkinElmer (Product No. NEX240050UC, Waltham, MA, USA).

The bombesin antagonist analog denoted NOTA-PEG6-RM26 (NOTA-PEG6-D-Phe-Gln-Trp-Ala-Val-Gly-His-Sta-Leu-NH2 ([D-Phe6, Sta13, Leu14] bombesin) [17] was synthesized and labeled with ^{111}In (Covidien, Dublin, Ireland) as described previously [16].

2.2. Cells

The human embryonic kidney 293 cell line (HEK-293) and the human prostate cancer cell line PC3 were used in this study. The cells were grown at 37 °C, 5% CO_2, in DMEM (HEK-293) or RPMI (PC3) medium containing 10% (v/v) fetal calf serum and PEST (penicillin 100 IU/ml and streptomycin 100 μg/ml). Additionally, the DMEM medium used for the HEK-293 cells contained Amphotericin B (Life Technologies, Carlsbad, CA, USA) and the RPMI medium for PC3 contained L-Glutamine (2 mM, Biochrom Ag, Berlin, Germany).

2.3. Transfection for transient and stable protein expression of hY2R

The hY2R was inserted into a pcDNA-DEST47 expression vector. HEK-293 cells were transfected with the hY2R expression vectors using Lipofectamine 2000 and OPTI-MEM according to the instructions from the manufacturer. The same transfection procedure was used for the stable transfection. After 24 h transfection, cells with stable expression were selected using DMEM medium containing 500 μg/ml geneticin. All reagents for transfection were from Life Technologies (Carlsbad, CA, USA).

2.4. Real-time measurement of interactions with GPCR on cells in LigandTracer Grey

Approximately 1 million cells were seeded in Petri dishes (Cat. No. 172958, Nunc, Roskilde, Denmark) at least 2 days before experimental day. All binding measurements were monitored in real-time on cells using LigandTracer Grey instruments (Ridgeview Instruments AB, Vänge, Sweden), essentially as described previously [12,13,18–20].

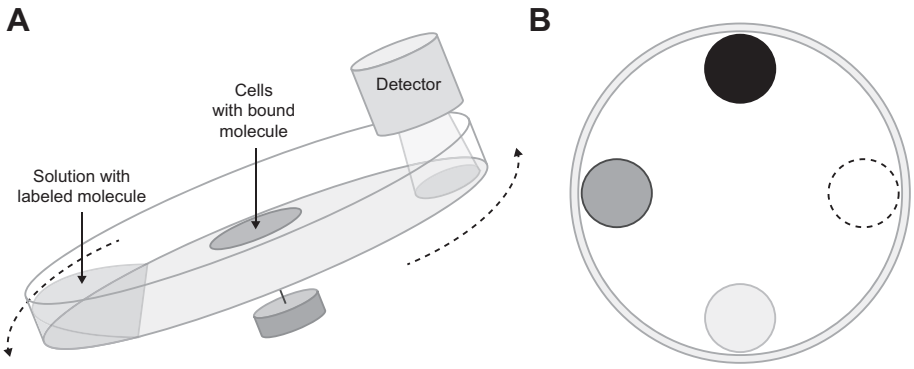

Fig. 1. Illustration of the LigandTracer technology and example of cell areas. (A) Cells are seeded in a local area of a Petri dish, which is placed on a tilted, rotating support. A solution with radiolabeled molecule is added. Radioactivity measurements are performed in the elevated area and followed over time. A target free area of the dish is used as a reference to continuously subtract the background signal. (B) In one of the experiments, the binding to three cell areas were monitored simultaneously: stably hY2R-transfected HEK-293 cells (black area), transiently transfected HEK-293 cells (dark grey area) and wild-type HEK-293 cells (light grey area). A plastic area (dashed circle) was kept cell free, used for background subtraction.