Application Note

Profiling the pharmacology of GPCRs by time-resolved impedance measurements

Topic:
GPCRs (Y4R, H1R)
Cells:
U373 endogen. H1R, CHO overexpr. Y4R
Tools:
AtlaZ

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Summary

G-protein-coupled receptors (GPCRs) are extensively studied drug targets in medicinal chemistry and pharmacology. The human genome encodes almost 900 GPCRs, which account for approximately 4 % of the genome, hence it's not surprising that they play roles in numerous physiological processes. It is estimated that about 40 % of all drugs act through GPCRs. Dysfunctions in these receptors have been linked to various severe diseases, including diabetes, allergies, depression, and certain forms of cancer, among others [1,2,3,4].

Traditional screening methods to study GPCR pharmacology involve genetically engineered cells with overexpressed receptors, focusing on ligand-GPCR binding and their downstream signaling. Non-invasive, label-free impedance measurements offer real-time monitoring of GPCR activation (agonist/antagonist mode) in target cells with endogenous receptor expression or engineered cells lines overexpressing the receptor. Heterotrimeric G-proteins transmit receptor activation into intracellular signaling cascades. Based on the amino acid sequences of their individual Gα-subunits (Gs, Gq, Gi/o, G12/13), a distinct signaling pathway is triggered. Here, we demonstrate the usefulness of impedance-based assays to unravel GPCR pharmacology by example of two of the three signaling pathways involving second messengers: (i) the Histamine H1 Receptor (H1R), which couples through the Gq-protein, and (ii) the Neuropeptide-Y Receptor 4 (Y4R), which couples through the Gi-protein. Unlike the other pathways, the G12/13 pathway does not directly modulate second messengers, like cAMP or IP_3 (Figure 1); instead, it leads to the activation of Rho family GTPases, such as RhoA, which regulate cytoskeletal dynamics, cell migration, and gene expression.

This Application Note demonstrates the *in vitro* characterization of GPCR pharmacology, covering agonist and antagonist mode of action, dose-response relationships, and the involvement of signal transduction cascades. Experimentally, cells expressing H1R or Y4R are cultured on planar gold-film electrodes integrated into standard cell culture dishes. Here, the planar gold-film electrodes embedded in a 96-well plate format are used as growth substrate for the adherent cells and as a sensor to monitor the cell response. H1R is a GPCR that is known to activate phospholipase C (PLC) via Gq, whereas Y4R belongs

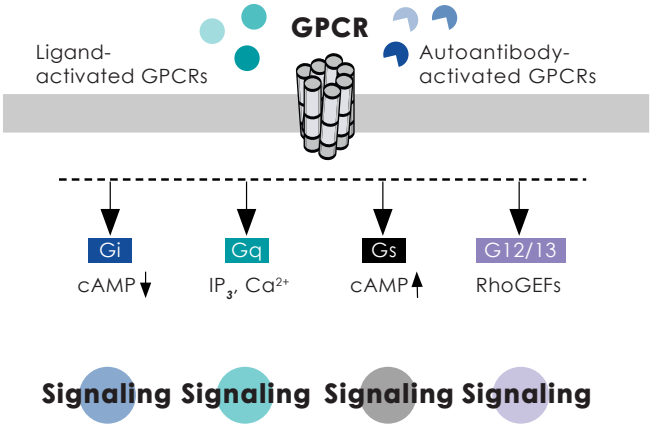


Figure 1: ***Why is label-free impedance sensing so attractive?*** *Different GPCR-dependent signaling pathways induce morphological changes of cells. Independent of which pathway being active, these changes can be measured via impedance.*

to the neuropeptide Y receptor family coupled to Gi.
For the Y4R, we used the endogenous agonists hPP (human pancreatic peptide) and the Synthetic Agonist 1 (SA1). CHO cells overexpressing the Y4R were exposed to these agonists. Impedance measurements as performed here reveal changes in cell morphology.

The strength of the AtlaZ assay approach with regards to GPCR as a target is its (i) time resolution, (ii) throughput, (iii) the fact that it is label-free and (iv) independent of genetic engineering. The technology is applicable to primary cultures or finite cell lines with endogenous receptor density [5]. A crucial advantage over standard assays that rely on labels for optical or radiometric detection, is the continuity of cell monitoring. Endpoint assays using dyes or labels just report on the status of the cells at a single time point but neglect the kinetics of the cell response.

Results

In this study, we used U373 cells endogenously expressing the Histamine H1 Receptor (H1R) or CHO cells overexpressing the Neuropeptide-Y Receptor 4 (Y4R).

U373 cells endogenously expressing H1R

U373 is a human glioblastoma astrocytoma cell line derived from a malignant tumor by explant technique. U373 cells were seeded in culture medium at a density of 100.000 cells/cm^2. Cell adhesion was monitored over the next 20 h (Figure 2A). Medium was exchanged 2 days after cell seeding. On day 3, U373 cells were stimulated with Histamine dissolved in L-15 medium (CO_2-independent). At the beginning of the assay, cells were allowed to adapt to fresh L-15 medium (100 µL/well) within 2 h prior to stimulation. The recording mode used here was "multiple frequencies" i.e. the impedance is recorded at several designated AC frequencies. Here, the impedance was recorded at 10 frequencies between 100 Hz and 100 kHz equally spaced on a logarithmic scale. The stock solution of the agonist Histamine (Histamine Dihydrochloride) was 20 mM in L-15 medium, stored at – 20 °C and diluted in L-15 medium according to the required working concentration. As a control experiment, the antagonist Mepyramine (Mepyramine Maleate, M = 401.5 g/mol; stock was 10 mM in L-15 medium, stored at – 20 °C) was added to wells 20 min prior to Histamine stimulation. Final concentrations of Histamine were 0 µM, 0.03 µM, 0.1 µM, 0.3 µM, 0.6 µM, 1 µM, 3 µM, 10 µM, 30 µM. In antagonist mode, the cells were pre-incubated with 0.03 µM Mepyramine for 20 min.
Data presented in Figure 2 B reveal a concentration-dependent response of the cells upon Histamine addition. The impedance increase mirrors the cell shape change that is associated with the intracellular, Gq-mediated signaling cascade. The impedance increases steadily after stimulation, runs through a maximum about 10 min after compound addition before it slowly returns to baseline conditions. This profile is similar to profiles observed earlier [6] and is a strong experimental indicator that the stimulation is predominantly mediated by the Ca^{2+}/IP_3 dependent signaling cascade. The EC_{50} value was estimated to be 689.5 nM (Figure 2 D). Mepyramine blocked or reduced the Histamine effect dependent on Histamine concentration (data not shown).

CHO cells overexpressing Y4R

The NPY receptor 4 is coupled to the Gi/o-protein that reduces the activity of the cAMP-producing enzyme adenylate cyclase. With cAMP concentrations being low in resting cells, the activation of the Gi/o pathway is experimentally hard to catch unless adenylate cyclase is experimentally stimulated before. A well-established, receptor-independent inducer of adenylate cyclase is forskolin. To find an appropriate forskolin concentration, CHO Y4R cells were exposed to 0.1 nM, 1 nM, 10 nM, 100 nM, 1 µM and 10 µM forskolin (Figure 3 A, B). To

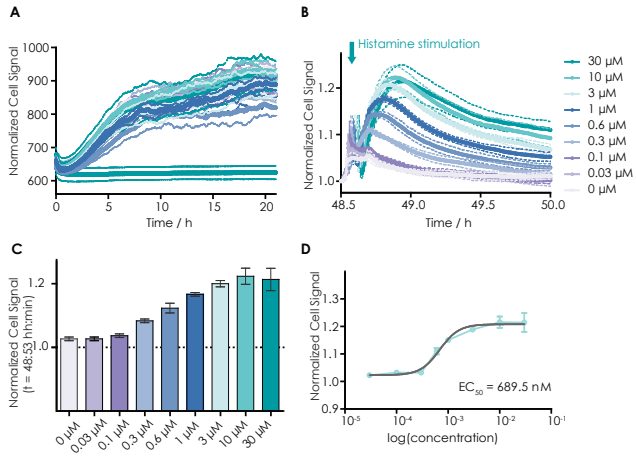
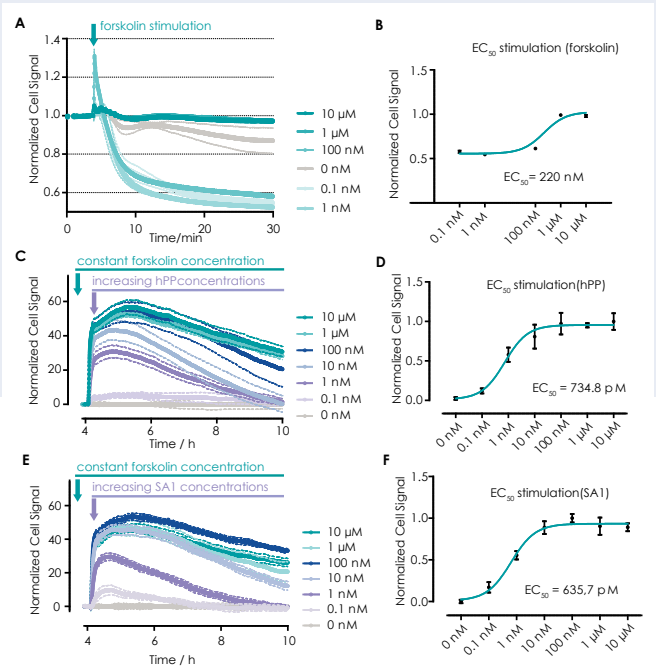


Figure 2: *Time-resolved impedance profiles of human glioblastoma cells (U373), (A) raw traces for adhesion and growth (seeding at time zero, n = 14 wells), (B) normalized traces for stimulation of U373 cells with Histamine at 0.03 µM - 30 µM (normalized to cell signal in L-15 medium before Histamine addition), (C) bar graph for normalized cell signal at t = 48:53 hh:mm. (D) EC_{50} estimated to be 690 nM. Data are shown +/- SD, n = 6 each.*

Figure 3: *Time-resolved impedance profiles of Y4R stimulation in CHO cells, (A) stimulation via increasing forskolin conc., (B) dose-response relationship yielding an EC_{50} of 220 nM. (C) Stimulation of Y4R with the endogenous ligand hPP, (D) dose-response relationship yielding an EC_{50} of 735 pM. (E) Stimulation of Y4R with increasing conc. of SA1, (F) dose-response relationship yielding an EC_{50} of 636 pM. Data are shown +/- SD, n = 6 each.*



identify the kinetics of forskolin effect, a high repetition interval of 1 s between sequential impedance readings was chosen (Figure 3 A). We estimated an EC_{50} of 220 nM (Figure 3 B), and the optimal forskolin concentration used in the following experiments was 400 nM, inducing a maximum effect. This concentration was applied to pre-stimulate CHO Y4R cells prior to the addition of the endogenous ligand hPP at 0.1 nM, 1 nM, 10 nM, 100 nM, 1 µM and 10 µM (Figure 3 C, D), or SA1 at 0.1 nM, 1 nM, 10 nM, 100 nM, 1 µM (Figure 3 E, F). The result of the dose-response analysis is given in Figures 3 D and 3 F, respectively. The CHO cell seeding density in these experiments was 100.000 cells/cm^2. The compounds were added once the cell layers were fully established. The impedance signal of the cell monolayer was recorded every 120 seconds for both, hPP and SA1, and is plotted using the unitless parameter Cell Signal (Figure 3). The time course of impedance revealed distinctly different profiles compared to the H1R in U373 cells, indicating that different signaling pathways may provide distinct impedance profiles. However, our data suggests this is not always true as it also depends on the origin of the tissues and cell density.

In conclusion, the AtlaZ system offers a highly automated approach capable of studying the pharmacology of various GPCR signaling pathways using a single platform. In addition, the AtlaZ system does not require labeled reagents and provides the cell response to receptor stimulation in real-time. The latter has proven its usefulness, for instance, when photoswitchable GPCR agonists [7] or receptor desensitization [8] have been studied. Thus, straightforward assay design, minimal artifacts, and short assay time, contribute to higher and more efficient throughput provided by the AtlaZ.

It is also important to emphasize that impedance-based monitoring of GPCR stimulation is sensitive enough to work with endogenously expressed receptors, like in primary cultures or even stem cells. The assays will provide the highest physiological relevance when no component of the signaling cascade is overexpressed. Moreover, it avoids the need for genetic engineering prior to receptor characterization and thereby reduces the biological safety label when conducting the assay.

Impedance-based cell monitoring is often called a *holistic approach* as the impedance integrates over the entire cell body without any molecular specificity as, for instance, provided by most label-based assays. As such, it provides an unbiased perspective on the cell under study. The lack of molecular specificity may require some extra careful assay procedures and controls to avoid false-positive results. But it unfolds its full strength when orphan receptors are being studied. The search for a yet unknown endogenous agonist typically goes along with no information on the coupling scheme of the receptor. Thus, an unbiased assay readout that is independent of the downstream signaling cascade but sensitive to all of them is the most efficient readout in de-orphanizing campaigns.

Methods

AtlaZ platform

The AtlaZ platform (Nanion) provides quantitative live-cell analysis by measuring the impedance (Ohm, Ω), displayed as Cell Signal, of adherent cells grown on 96-well plates with embedded planar gold-film electrodes (Nanion). The methodology, Electrical Impedance Spectroscopy (EIS), provides a unique richness of information from cells. Depending

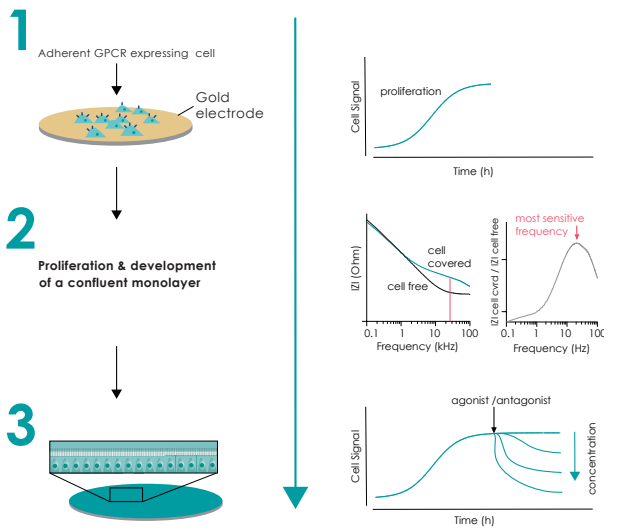


Figure 4: *Workflow of a GPCR assay.* *Target cells adhere and proliferate (<= 48 h) (1). Upon reaching a plateau phase, spectra for cell-free and cell-covered electrodes reveal the most sensitive frequency (2) by plotting the ratio of the impedance magnitude |Z| of electrodes with cells and the impedance |Z| of electrodes without. Next, the treatment can be executed (3). The Cell Signal is monitored continuously over time, revealing the kinetics of stimulus-induced effects.*

on the data acquisition frequency, the system detects predominantly the resistive part of cell-cell and cell-matrix contacts at lower frequencies or predominantly capacitive currents across the cell membranes at higher frequencies as an indicator for electrode coverage. The latter is the basis for cell adhesion or migration assays. Thus, real-time impedance data provide insights into various cell phenotypes, such as cell morphology changes as a result of signaling, proliferation, lateral migration or cytotoxicity even over prolonged periods of time.

GPCR assay

Here, we used the CHO cell line which has been transfected to overexpress a GPCR (CHO-Y4, Y4R) or a finite cell line with endogenous GPCR expression (U373, H1R). The readout, displayed as the Cell Signal, represents raw impedance values. For these specific recordings we have chosen a 12 kHz recording frequency, which is best suited for these type of pathway investigations. The optimal frequency is cell-type specific and has to be determined empirically, but for most electrode types it ranges between 1 kHz and 50 kHz [9]. As a general guideline, the sensitive frequency for detection of changes in cell layer properties can be determined by dividing the impedance magnitude |Z| of a cell-covered electrode by the impedance of a cell-free electrode along the frequency spectrum (Figure 4). When these ratios are plotted for each individual frequency, one obtains a bell-shaped curve from which the maximum ratio reflects the frequency with the broadest range of relative change in impedance (Figure 4), i.e. the most sensitive frequency.

Key findings

- Our results demonstrate the capability of AtlaZ to characterize the pharmacology of GPCRs and monitor major G-protein-dependent pathways.
- AtlaZ allows for cellular research on cell adhesion, proliferation, cytotoxicity and GPCR-mediated signal transduction effects, label-free and in real-time.
- Recordings can be performed for up to 6 x 96 = 576 samples either simultaneously or independently.

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