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Impedance-based analysis of mu opioid receptor signaling and underlying mechanisms

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ABSTRACT

The mu opioid receptor is a G-protein coupled receptor able to signal through the $G\alpha_{i/o}$ class of G-protein and β-arrestin pathways, stimulating down-stream effector pathways. Signaling bias occurs when different receptor agonists lead to different signaling outcomes. Traditionally these have been studied using end-point assays. Real-time cellular analysis platforms allow for the analysis of the holistic effects of receptor activation as an integrated output. While this allows for different ligands to be compared rapidly, the cellular mechanisms underlying the signal are not well described. Using an impedance based system, the impedance responses for two opioid ligands, morphine and DAMGO were examined.

The impedance responses for these two agonists, while showing similar features, were distinct from each other. Some of the mechanisms underlying the mu opioid receptor coupled impedance changes were investigated. It was found that the response is a result of discrete cellular processes, including G-protein signaling and protein kinase phosphorylation.

1. Introduction

The mu opioid receptor (MOR) is a member of the opioid family of receptors and the large G-protein coupled receptor (GPCR) superfamily. Activation of this receptor results in a GDP/GTP exchange at specific G-proteins with the resulting release of the G-protein subunits leading to stimulation of downstream effector pathways. Receptor activation can also result in the recruitment of β-arrestin leading to receptor internalisation and the activation of a G-protein independent signaling pathway. Agonists can activate different pathway subsets leading to signaling bias [1,2].

The opioid agonists [D-Ala^2, NMe-Phe^4, Gly-ol^5]-enkephalin (DAMGO) and morphine are both MOR agonists which have been shown to exhibit bias in their signaling [3,4]. One measure of this bias is the induction of receptor internalisation. In the majority of cell types, treatment with DAMGO leads to internalisation while morphine shows relatively less internalisation [5–8]. A second illustration of bias is the observation that in β-arrestin2 knockout mice, morphine analgesia is enhanced while respiratory depression is reduced [9].

In addition to ligand bias changing the level at which pathways can be activated, the outcomes of receptor stimulation can be influenced by type or state of a cell. There is also the possibility of unexpected pathways being activated causing the full set of effects to be missed [3,5,6,10,11]. A system with a read-out capturing the whole cell response to receptor activation will allow a holistic comparison of agonists. Label-free real-time cellular analysis (RTCA) platforms allow an approach naïve to the actual mechanisms activated but instead capture an integrated whole-cell output. This can include cell processes that have not previously been described [11,12].

One RTCA platform uses an impedance based assay, which measures the change in impedance over time produced by cultured cells grown on an array of interdigitated circuits on the base of a 96-well microplate. This provides an output integrating the whole cell signals that lead to a change in impedance resulting from ligand stimulation. The assay is label-free and provides for a real-time analysis of cell events [13,14]. This technique is starting to be utilised to examine GPCR ligands with a goal of allowing an unbiased method of classifying ligands into discrete pharmacological categories and to give an indication of their signaling bias [12,15]. Most of the underlying mechanisms that lead to ligand-induced cellular morphological changes and a change in cellular impedance are not well understood. A better understanding of what cellular processes underlie impedance profiles will allow a more confident assessment of compounds and the prediction of their properties. This may have applications such as the screening of compounds for desirable properties.

Here the impedance profiles generated by the opioid agonists DAMGO and morphine were compared using Chinese Hamster Ovary (CHO) cells stably overexpressing the MOR and some of the cellular processes behind the impedance response were investigated. In particular the contribution of kinase signaling cascades to the response

Abbreviations: MOR, mu opioid receptor; GPCR, G-protein coupled receptor; CI, cell index; RTCA, real-time cellular analysis; CHO, Chinese hamster ovary; FCS, foetal calf serum; DAMGO, [D-Ala^2, NMe-Phe^4, Gly-ol^5]-enkephalin

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was examined. The results showed that individual opioid agonists can lead to distinct impedance profiles. The impedance response is the result of discrete cellular processes which act in a time-dependent manner. Part of this response is due to the contributions of the AKT1/2/3 pathway, in the early stages of the response and the ERK1/2 pathway at later stages of the response.

2. Material and methods

2.1. Cell culture

CHO cells stably expressing the human MOR were a gift from Dr. Meritxell Canals [16]. Cell culture medium and foetal calf serum were purchased from Gibco. Cells were maintained in DMEM medium supplemented with 4.5 g/l D-Glucose and 10% foetal calf serum (FCS) and incubated in a humidified incubator at 37 °C with 5% CO_2.

2.2. Impedance measurement

The xCELLigence system (ACEA Biosciences, San Diego) was used to measure changes in cellular impedance resulting from stimulation with a ligand in 96-well E-Plates [13]. The E-Plate has electrode arrays integrated into the bottom of the wells which allow for the measurement of the impedance conferred by cells growing on this surface. Changes in the measured impedance are defined by a unit-less cell index (CI). Prior to the addition of cells to the E-Plate, a CI measurement was taken in the presence of 100 µl growth medium to determine the background CI value for each well. This was subtracted from the subsequent CI values as they were collected following cell attachment. Cells were seeded at a density of 2×10^4 cells/well and cultured overnight in growth medium. After approximately 24 h, the cell medium was replaced with serum free DMEM and the volume of medium in the wells was adjusted to 180 µl for assays only requiring ligand or 160 µl for assays requiring an additional treatment and incubated overnight for approximately 16 h.

The E-plate was kept in the incubator for the duration of the experiments. For the addition of compounds, E-Plate lid was replaced with the RTCA Protector Shield 96 (allowing access to the wells whilst the E-Plate is actively monitored in the cradle) and the E-Plate was equilibrated for 30 min. Compounds were diluted in serum-free medium to a 10× concentration, and added using an automated multichannel pipette (20 µl /well). For agonist addition, the sampling frequency was increased to the maximum possible with CI values collected at 10 s intervals for a minimum period of 1 h.

For analysis, CI values were normalised by dividing by the cell index at the time immediately prior to ligand addition (time = 0 min). Baseline corrections were carried out by subtracting the cell index obtained from vehicle-treated cells. Between four and eight replicates were used for each treatment per experiment.

2.3. Kinase activation assay

Cells were plated and grown in 96-well plates at a density of 4×10^4 cells/well. The cells were cultured and treated with ligands as described for the impedance measurement assay. ERK1/2 and AKT1/2/3 phosphorylation were measured using the ERK1/2 (Thr202/Tyr204), AKT1/2/3 Thr308 and AKT1/2/3 Ser473 AlphaScreen Surefire kit (Perkin Elmer). Cells were treated with 10 µM of either DAMGO or morphine for varying periods of time required for the experiment, then immediately lysed in 100 µl of the supplied lysis buffer with shaking for 10 min at room temperature. Plates were then sealed and stored at −20 °C. After thawing on ice, 5 µl were transferred to a 384-well ProxiPlate (Perkin Elmer) to be used for the phosphorylation assay as per the manufacturer's instructions.

2.4. Data analysis

Graphing and analysis of results were performed using RTCA software (v1.2.1.1002) provided by Roche Applied Science with the xCELLigence System and Prism5 (GraphPad, CA, USA).

2.5. Materials

U0126, GSK2334470 and FB-124 were purchased from Tocris, DAMGO [D-Ala^2, NMe-Phe^4, Gly-ol^5]-enkephalin) and Morphine sulphate were purchased from Sigma.

3. Results and discussion

3.1. Impedance profiles

Real-time measurement of impedance results in a profile which represents the integration of multiple signal events stimulated by receptor activation. The MOR is known to activate two signaling pathways: $G\alpha_{i/o}/G\beta\gamma$ and β-arrestin. To determine the profiles resulting from opioid receptor stimulation and to investigate whether impedance measurements can distinguish between different agonists, CHO cells stably overexpressing the human MOR were treated with either DAMGO or Morphine.

The stimulation of cells using each agonist leads to a concentration-dependent, multi-featured, impedance response (Fig. 1). The treatment of cells with either DAMGO or morphine resulted in profiles that displayed broadly similar features, although each agonist displayed unique characteristics (Fig. 1A). The impedance profiles could be divided into several phases. Stimulation using both agonists resulted in a rapid rise in CI (rapid ascending phase), a peak (major peak) and then an initial decay (first decay). This was followed by a "second ascending phase" and a second peak (minor peak). Finally a "second decay" lead to a "plateau phase" in which the CI is maintained at a level higher than the initial baseline.

The rapid ascending phase and major peak for each agonist showed a high degree of similarity. In contrast, the region of the profile representing the minor peak showed differences. In particular, the CI of this region was higher for cells stimulated by morphine compared to DAMGO.

To demonstrate that this response was due to MOR activation, cells were treated with the pan-antagonist naloxone (10 µM) prior to agonist addition. Treatment with naloxone resulted in the complete inhibition of the impedance response of both DAMGO and morphine (Fig. 1B and C). Furthermore, the dose-response curve elicited by both agonists was moved to the right with increasing concentrations of Naloxone, indicating a competitive antagonism (data not shown).

Previous studies have examined the impedance response elicited by the activation of several GPCR's and have shown a diverse range of impedance profiles. These include activation of the β_2-Adrenergic receptor, the niacin receptor GPR109A and the chemokine receptor CXCR3 [12,15,17]. These reports showed that the stimulation of each of these receptors resulted in a specific changes in cellular impedance and that the response for an individual receptor was dependent on the agonist used. The impedance profiles generated by the stimulation of the MOR were distinct from those previously published, although there were similarities with the two other $G_{i/o}$ coupled receptors, GPR109A and CXCR3. All three of these receptors have profiles which contain a feature similar to the major peak shown here.

The impedance profiles were concentration-dependent with different features of the impedance profile showing differential responses (Fig. 1D and E). This indicates that separate signaling events were involved in the development of the impedance responses over time. This was demonstrated by generating dose-response curves for two of the features of the profile; the major and minor peaks