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Advantages and shortcomings of cell-based electrical impedance measurements as a GPCR drug discovery tool

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ABSTRACT

G Protein-Coupled Receptors (GPCRs) transduce extracellular signals and activate intracellular pathways, usually through activating associated G proteins. Due to their involvement in many human diseases, they are recognized worldwide as valuable drug targets.

Many experimental approaches help identify small molecules that target GPCRs, including *in vitro* cell-based reporter assays and binding studies. Most cell-based assays use one signaling pathway or reporter as an assay readout. Moreover, they often require cell labeling or the integration of reporter systems.

Over the last decades, cell-based electrical impedance biosensors have been explored for drug discovery. This label-free method holds many advantages over other cellular assays in GPCR research. The technology requires no cell manipulation and offers real-time kinetic measurements of receptor-mediated cellular changes. Instead of measuring the activity of a single reporter, the impedance readout includes information on multiple signaling events. This is beneficial when screening for ligands targeting orphan GPCRs since the signaling cascade(s) of the majority of these receptors are unknown. Due to its sensitivity, the method also applies to cellular models more relevant to disease, including patient-derived cell cultures.

Despite its advantages, remaining issues regarding data comparability and interpretability has limited implementation of cell-based electrical impedance (CEI) in drug discovery. Future optimization must include both full exploitation of CEI response data using various ways of analysis as well as further exploration of its potential to detect biased activities early on in drug discovery.

Here, we review the contribution of CEI technology to GPCR research, discuss its comparative benefits, and provide recommendations.

1. Introduction

1.1. G protein-coupled receptor signaling

In humans, and other animals, GPCRs form the largest superfamily of cell surface receptors involved in transmembrane signaling. GPCRs can transmit signals into the cell by responding to a highly diverse set of extracellular stimuli, such as, polypeptides, glycoproteins and ions, thereby regulating a wide range of physiological and developmental functions (Dorsam and Gutkind, 2007; Syrovatkina et al., 2016). Given the broad range of biological processes in which GPCRs are involved, deregulated GPCR signaling contributes to many pathophysiological conditions, in many different disease areas (Gutierrez and McDonald, 2018; Heng et al., 2013; Schöneberg et al., 2004). This contribution, together with the ability to modulate GPCRs pharmacologically, makes GPCRs the most commonly exploited category of drug targets (Nambi and Aiyar, 2003; Santos et al., 2017) with currently 20–30% of all FDA-approved drugs targeting GPCRs (Rask-Andersen et al., 2011; Wacker et al., 2017). The disease areas in which GPCRs have been validated or are considered as promising therapeutic targets include metabolic and cardiovascular diseases (*e.g.*, obesity, type 2 diabetis) (Heng et al., 2013), neurodegenerative diseases (*e.g.*, Alzheimer's, Parkinson's, Huntington's disease) (Huang et al., 2017), immunological disorders and inflammation (Nijmeijer et al., 2016; Viola and Luster, 2008), infectious diseases (*e.g.* HIV) (Brelot and Chakrabarti, 2018; Zaitseva et al., 2003) and various forms of cancer (Bar-Shavit et al., 2016;

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Dorsam and Gutkind, 2007; Gutierrez and McDonald, 2018).

Intracellular signaling cascades activated by GPCRs can be quite complex. When a ligand occupies its GPCR binding pocket, conformational changes in the receptor occur. These changes promote binding of heterotrimeric G proteins consisting of G_{α}-GDP and $G_{\beta\gamma}$-subunits at the intracellular receptor part. Exchange of GTP for GDP on the G_{α}-subunit leads to the reversible dissociation of the G protein subunits, initiating downstream signaling via G_{α}-GTP and $G_{\beta\gamma}$ (Neer, 1995). The human genome encodes 16 different G_{α}-subunits, 5 G_{β}-subunits and 12 G_{γ}-subunits (Downes and Gautam, 1999). $G_{\beta\gamma}$-subunits are assumed to be functionally interchangeable (Smrcka, 2008). In contrast, G_{α}-subunits have clearly dinstinct properties, as they can engage with dinstinct effector molecules and induce defined intracellular signaling pathways (Marinissen and Gutkind, 2001). GPCRs are often divided into four distinct categories of G_{α}-coupling: G_s, $G_{i/o}$, $G_{q/11}$, and $G_{12/13}$ (Hepler and Gilman, 1992). As first evidenced in the early eighties (Asano et al., 1984), it is now widely accepted that some GPCRs can couple to multiple G_{α} proteins (Campbell and Smrcka, 2018; Hermans, 2003).

Upon receptor activation, β-arrestin molecules will be recruited to the phosphorylated receptor tail, thereby not only terminating G protein-signaling, but also enhancing receptor internalization and recycling (Penela et al., 2003; Pitcher et al., 1998). In the late nineties, it was evidenced that β-arrestins are also capable of triggering signaling cascades independent of G protein-signaling, such as MAPK activation (Daaka et al., 1998; Luttrell et al., 1997). It has been widely accepted that β-arrestin dependent events account for an additional layer of GPCR signaling complexity (Ahn et al., 2004; DeWire et al., 2007; Heuss and Gerber, 2000; Lefkowitz and Shenoy, 2005) and that interfering with β-arrestin driven processes could offer novel strategies for therapeutic intervention as well (Bond et al., 2019; Laporte and Scott, 2019). However, β-arrestin dependent signaling, more specifically the G protein independence of MAPK signaling, was recently challenged using G_{α} protein and β-arrestin knockout in Human Embryonic Kidney (HEK293) cells (Grundmann et al., 2018). Also, since there is evidence that GPCRs can signal via G proteins on endosomes (Irannejad et al., 2013) and since this wave of signaling could vary depending on the amount of β-arrestin mediated internalization, changing β-arrestin levels could influence G protein-signaling which potentially has been misinterpreted as being G protein-independent (Campbell and Smrcka, 2018).

GPCR signaling generally results in changes in cellular morphology through modulation of the actin cytoskeleton (Dutt et al., 2002; Singh et al., 2007). Different signaling pathways can alter the cellular morphology in a specific manner. Activation of G_i-coupled receptors (Saltarelli, 1999; Schraufstatter et al., 2001) and G_q-coupled receptors (Kawabata et al., 1999; Pierce et al., 1999) leads to enhanced actin polymerization (actin stress fiber formation), while G_s stimulation leads to actin depolymerization (Perez et al., 2005). Also, other cytoskeleton proteins, such as spectrin, have been associated with GPCR-induced changes in morphology (Street et al., 2006). Via cytoskeleton rearrangements, GPCRs also induce cellular movement or cell migration which is well known for the chemokine receptor family (Griffith et al., 2014; Stein and Nombela-Arrieta, 2005). Besides indirect modulation of the cytoskeleton downstream canonical GPCR signaling, evidence also exists for a more direct filamin-dependent coupling between the GPCR and cytoskeleton (Tirupula et al., 2015).

1.2. GPCR drug discovery: one goal, many assays

GPCR drug discovery is a costly and time consuming process that in the initial stage mainly relies on high-throughput screening (HTS) of small molecule compounds on heterologous cells overexpressing the GPCR of interest (Takakura et al., 2015). Second messengers or coupling with intracellular effectors are usually employed as the readout for these screens. Hits are confirmed by thorough efficacy and affinity testing during the hit-to-lead (H2L) stage while also predictions are made regarding addition, absorption, distribution, metabolism and excretion (ADME) of the hits. Next, lead optimization occurs, in which selected leads are optimized by structure-activity relationship (SAR) studies. The optimized leads then undergo efficacy and safety testing *in vivo* while also ADME is further evaluated. After thorough selection and optimization the most promising candidates will eventually move on to clinical trials (Kumari et al., 2015). HTS for GPCR ligands, however, often fails to deliver ligands that are suitable for subsequent optimization. The strong focus on potency during HTS often leads to candidates with relatively high MW and suboptimal physiochemistry, features that hamper lead optimization (Lipinski et al., 1997). Moreover, drug potency obtained on heterologous overexpression cell lines often do not resemble *in vivo* potency. As such, novel strategies that allow the use of more relevant cell types early on in drug discovery are desirable.

Currently, ~110 GPCRs, which account for one third of the potentially druggable GPCRs, are targeted by ~475 FDA-approved GPCR-targeting drugs (Hauser et al., 2018; Sriram and Insel, 2018). At the late preclinical stage, about 100 GPCRs are currently being evaluated as drug targets, whereby ~400 small molecule drugs are being investigated (Wacker et al., 2017). Most GPCR targeting drugs under study target well-validated GPCRs (Lafferty-Whyte et al., 2016; Wacker et al., 2017) while almost no orphan receptors are being evaluated as therapeutic targets (Roth and Kroeze, 2015). Moreover, some groups such as the adhesion GPCRs, potentially important targets for neurological diseases (Folts et al., 2019), have no small molecules in clinical testing so far (Lafferty-Whyte et al., 2016). The absence of many of these GPCRs in clinical testing is partly due to a lack of robust and scalable assays to assess their activities (Sriram and Insel, 2018).

1.2.1. Label-based functional assays

The readout of GPCR label-based functional assays is typically limited to one particular signaling pathway, which is activated by the receptor under study (Thomsen et al., 2005). Second messenger assays are classic examples of this, including monitoring the transient mobilization of Ca^{2+} from intracellular stores for G_q-coupled receptors, or analyzing changes in the level of cyclic AMP for G_s and G_i coupled receptors (Zhang and Xie, 2012). Although these assays have proven successful in identifying GPCR ligands and are often used for HTS, they share several limitations. All these assays require cell labeling or genetic engineering (*e.g.*, reporter assays) to evaluate receptor activity (Fang et al., 2008). Also, when the natural ligand for a receptor is unknown (orphan GPCR), so are often the corresponding receptor-mediated signaling pathways, thus excluding the use of many traditional cell-based assays.

Some assays are applicable to a broader range of GPCRs (Inoue et al., 2012; Jacobson, 2015; Mertens et al., 2004; van Der Lee et al., 2008). For example, co-transfecting the receptor of interest and a promiscuous $G_{\alpha 15}$ or $G_{\alpha 16}$ makes it possible to screen orphan GPCRs using the calcium mobilization assay even when the receptor is not G_q-coupled (Caers et al., 2014; Offermans and Simon, 1995). Unfortunately, not all receptors couple with these promiscuous G proteins. Monitoring the recruitment of β-arrestin molecules upon receptor activation can also be employed as a more generic screening assay since β-arrestin recruitment is common to most GPCRs (Galinski et al., 2018; van Der Lee et al., 2008). Other examples are the β-arrestin redistribution assay and receptor trafficking assay (Zhu et al., 2014). However, in case of the latter, some ligands might not induce receptor internalization and will thus be overlooked. Also, the use of designer receptors exclusively activated by designer drugs (DREADDs) has shown great potential for GPCR-based drug discovery as well (Lee et al., 2014).

Besides cell-based screening approaches, structure- and sensor-surface based ligand discovery for GPCRs are also emerging as promising tools for GPCR drug discovery (Jacobson, 2015; Kumari et al., 2015). These alternative screening approaches are made possible due to the availability of high resolution GPCR crystal structures and advances in stable protein preparation i.e. isolation of stable functional recombinant

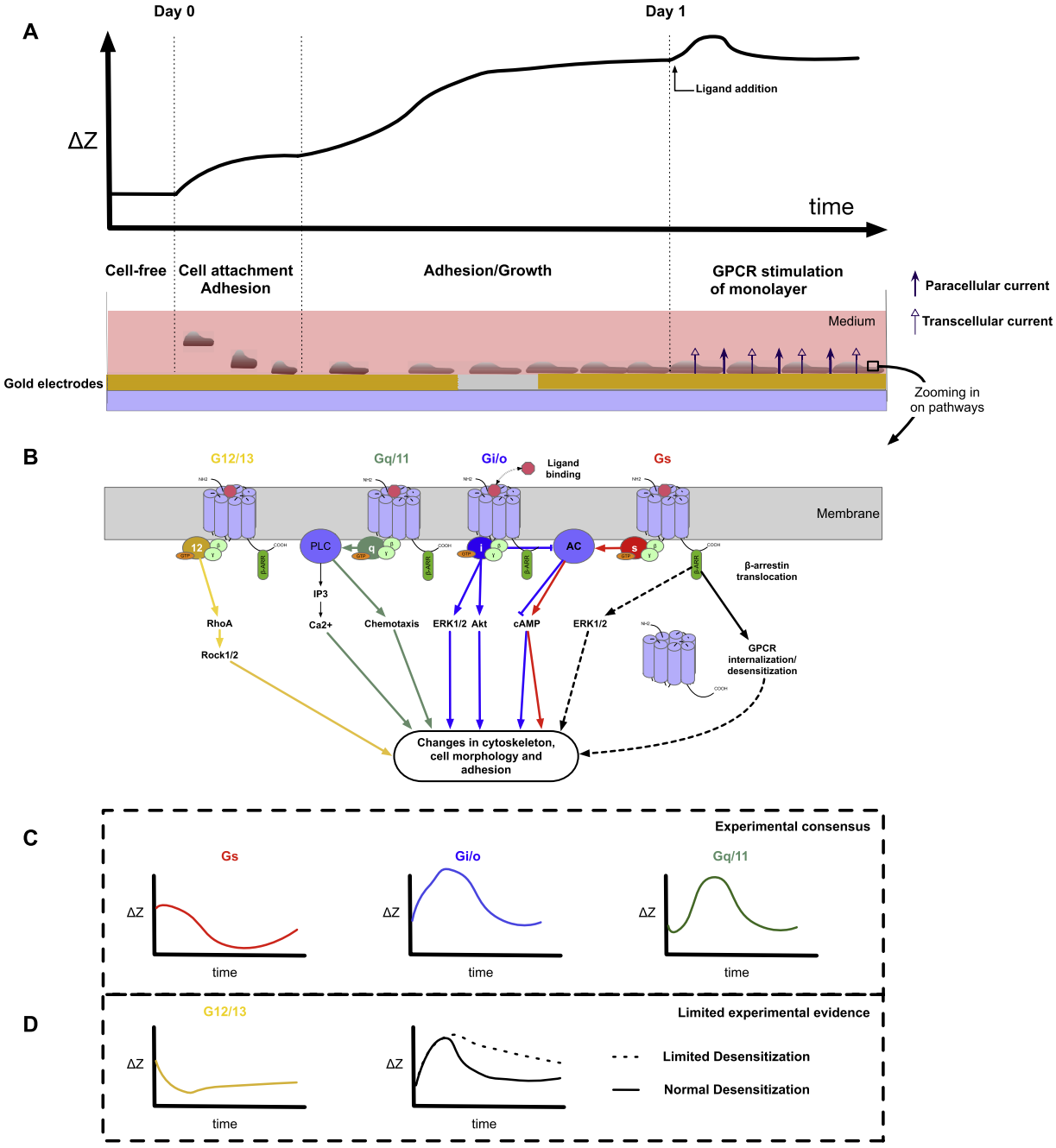

Fig. 1. Schematic representation of the different stages of a GPCR CEI assay, the pathways involved in GPCR-mediated impedance changes and the resulting CEI signatures. **(A)** CEI assay overview: The GPCR ligand is usually applied on day 1 when the cells have formed a monolayer. AC currents can run in paracellular and/or transcellular fashion depending on the applied frequency of AC flow. **(B)** Simplified representation of how ligand-induced GPCR signaling can lead to cellular changes detectable by CEI. It has been demonstrated that signaling via either one of the four major G_α protein classes ($G_{12/13}$: Yellow, $G_{q/11}$: Green, G_s: Red, $G_{i/o}$: Blue) results in characteristic changes in the CEI as overviewed in Table S1. **(C)** The CEI responses given for the major G_α protein classes G_s, $G_{i/o}$ and $G_{q/11}$ are inferred from Table S1 and point out often reoccurring features for each coupling. However, these profiles are not representative for all receptors, cell types, concentrations or readout frequencies. **(D)** CEI responses elicited by $G_{12/13}$ signaling remain understudied and as such the depicted profile is based on limited experimental evidence. Also, limited reports suggest that β-arrestin-mediated processes such as receptor desensitization can alter the kinetics of G_α protein-dependent CEI changes.

GPCRs.

1.2.2. Label-free cell-based technologies

Label-free technologies are based on the detection of changes in cell adhesion, cell shape, cell-cell interactions and/or viability which typically result from cytoskeleton changes upon ligand-induced GPCR activation (Doller, 2016). Label-free systems exploit these cellular changes as integrated non-pathway specific readouts. Whole-cell sensing label-free biosensors encompass optical- and electrical biosensors. Optical biosensors include resonant waveguide grating (RWG) and surface plasmon resonance (SPR). They can measure dynamic mass redistributions (DMR) from within the adherent portion of cells (up to 200 nm) near the well plate's base by detecting shifts in resonant wavelength and angle of reflected light. DMR results from cytoskeleton changes which typically occur downstream GPCR activation (Doller, 2016; Fang et al., 2006). For good reviews on optical biosensors for

GPCR research and for reports discussing label-free biosensors in general we refer to a selected list of papers (Fang, 2011, 2007; Lieb et al., 2016; Peters et al., 2010; Scott and Peters, 2010). Electrical or impedance-based biosensors employ a gold microelectrode array to measure changes in impedance of a cell layer that is exposed to an electric field. Cytoskeletal changes that occur in the cells upon GPCR activation will alter the cell layer's characteristics, which in turn will influence the measured impedance. Although currently underexplored in GPCR drug discovery, this technique represents an alternative approach to decipher GPCR activity and function, and to evaluate the activity of compounds, with potential advantages over label-based assays. In the following, we will critically review its usage, benefits and current shortcomings in the field of GPCR research.

1.3. Cellular electrical impedance – the basics

Electrical biosensors measure the impedance (Z) of cells that grow on top of a surface with embedded (gold) electrodes as the cells are exposed to an electric field generated by continuous sweeping of alternating current (AC) voltages over a range of frequencies. The measured impedance is the AC equivalent of direct current resistance (R) and contains both a resistance and reactance component. Because cells act as insulating particles when they are on top of the electrodes, they will impede the flow of the current and, hence, will increase the impedance of the system. As such, the impedance depends on the amount of cells present on the electrode surface (cell number, shape and size), on the cell's adhesion (distance between the cells and the electrode surface) and the cell-cell contacts (Doller, 2016). As multiple signaling events downstream GPCR activation can lead to changes in cell adhesion, morphology and cell-cell contacts, impedance responses are integrated in nature.

It was first shown that changes in cell attachment and spreading could be detected using this technique (Giaever and Keese, 1984). Later, the method was used to monitor cell motions as small as 1 nm (Giaever and Keese, 1991). The ability to monitor changes in morphology of adherent cells led to the development of several cell-based electrical impedance platforms by different manufacturers over the past decades. Besides some larger platforms, less-used platforms have been developed as well, but these are beyond the scope of our review.

The ECIS™ system (Applied Biophysics, NY, USA) was developed by Giaever and Keese (Giaever and Keese, 1993, 1984). It can monitor impedance changes over a range of frequencies between 25 Hz and 100 kHz. The xCELLigence™ instrument (ACEA biosciences, San Diego, USA) monitors the Cell Index (CI), a tractable statistic derived from the frequency-dependent resistance R from the complex impedance at 10, 25 and 50 kHz (Solly et al., 2004; Yu et al., 2006). In this manner, the readout frequencies are predefined. The CellKey™ system (Molecular Devices, CA, USA) also measures the impedance over a broad range of frequencies. Both CellKey and ECIS make a distinction between low frequency currents (< 2000 Hz) that mainly flow between the cells (*i.e.,* paracellular) and higher frequency currents (> 40.000 Hz) that can pass through the cells (*i.e.,* transcellular) more easily. Low frequency currents might be more sensitive to changes in cell-cell and cell-substrate contacts whereas high frequency currents could be more sensitive to changes in cell density (Leung et al., 2005; Miyano et al., 2014). The CellKey system was specifically designed for GPCR research but the platform is no longer commercially available.

Fig. 1A gives a schematic overview of the impedance assay for GPCR research and indicates how the impedance changes over the different stages of the assay, ranging from cell seeding to the cellular response elicited by a GPCR stimulus.

2. CEI: a versatile tool for GPCR research

2.1. The first steps towards cellular electrical impedance in GPCR research

In 1992, Tirrupathi and colleagues demonstrated how CEI can be used to study endothelial barrier function (Tiruppathi et al., 1992). Bovine Pulmonary Microvessel Endothelial Cells (BPMVEC), characterized by strong cell-cell and cell-substrate contacts, were exposed to thrombin. This caused a transient decrease in electrical resistance resulting from a temporary drop in endothelial barrier function. This method proved to be more convenient than filter-based permeability assays using ^{125}I-albumin, as it provides better temporal resolution while it also enables measuring the recovery process. Stolwijk et al. (2015) comprehensively reviewed impedance-based barrier function measurements and discuss necessary considerations when using this method, which has now become a routine assay for *in vitro* vascular barrier function (Stolwijk et al., 2015). Barrier function studies have been performed successfully both with the ECIS system and the xCELLigence system even though often performed at different readout frequencies (Kustermann et al., 2014; Rahim and Üren, 2011; Stolwijk et al., 2015).

Because thrombin and other molecules that affect barrier function (*e.g.*, histamine) induce GPCR signaling, several research groups started focusing on measuring the effect of GPCR signaling using CEI. In 1999, the endogenously expressed β2-adrenergic receptor was stimulated with isoprotenerol in Bovine Aortic Endothelial Cells (BAEC) and the impedance profile was recorded with a device based on the ECIS platform (Wegener et al., 1999). It was shown that alprenonol could block the impedance change. Since then, many different GPCRs have been studied using CEI, as discussed in the next section.

2.2. CEI is applicable to most GPCR types

Both recombinant cell lines with high levels of heterologous GPCR expression, as well as adherent cells with endogenous GPCRs expressed in their native environment (*e.g.*, immortalized cell lines, primary cells) have been used for CEI measurements (McGuinness, 2007; Yu et al., 2006). Supplementary Table S1 presents an overview of most GPCRs that have been studied so far with CEI, representing GPCRs that can signal intracellularly via different types of G_α proteins ($G_{i/o}$, $G_{q/11}$, G_s and $G_{12/13}$). The table also indicates the ligands that were used, in which cell type the GPCR was studied and if the GPCR was overexpressed or endogenously expressed. A few exceptions aside, most of the GPCRs monitored with CEI have also been thoroughly characterized by other cell-based assays, which allows the cross-validation of CEI with more conventional assays. From Table S1 it becomes clear that the vast majority of studied GPCRs belong to the Rhodopsin class A receptors. Basically, all class A subfamilies are represented in the table. Only few class B1 secretin members and few class C members (metabotropic glutamate receptors) have been studied with CEI so far. To our knowledge, only one class F (Frizzled) member and no GPCRs from class B2 or E have been investigated using CEI. The broad variety of GPCR types that can be assessed using CEI demonstrates its potential for GPCR drug discovery.

2.3. Ligand efficacy and potency determination and how it compares to traditional assays

It should be noted that it is often not easy to directly compare potencies between different functional assays as they often employ different incubation periods, are sometimes performed on different cell types, while also they focus on different steps in the signal transduction cascade meaning that the amplification factor and ceiling in each of the steps complicate the comparison. To determine potency and efficacy based on CEI responses, one mostly relies on peak values (maxima or minima) of the impedance response (Watts et al., 2012), or on the area

under the curve within a certain period during the assay (Doijen et al., 2017). However, activation of GPCRs can result in complex kinetic CEI profiles possibly with multiple maxima and/or minima, meaning that the considered timeframe and method for potency and efficacy calculation can strongly influence these values. For example, Stallaert and colleagues demonstrated for the β2-adrenergic receptor in transfected HEK293 cells that distinct potencies were obtained for maximal impedance response and the rapid ascending phase slope of the impedance response (Stallaert et al., 2012). Moreover, potency values determined from CEI might differ from other assays because activating signaling events as well as downregulating events can contribute simultaneously to the overall CEI response. This also means that a more overall potency predicition is obtained from CEI measurements than from many other assays.

Nevertheless, potency and efficacy values have been compared between CEI and various other cell-based assays. Most reports demonstrate that the rank order of potency derived from CEI experiments is highly comparable to that obtained in more traditional receptor assays. For example, the CellKey system yielded similar potency rankings for agonists of the endogenous purinergic P2Y receptor in U-937 cells when compared to those obtained using a calcium mobilization assay (Verdonk et al., 2006). Also, similar rank order of potency was obtained from the peak CEI response of niacin and various other compounds acting on GPR109A expressed in HEK293 cells than from the inhibition of forskolin-induced cAMP production (Kammermann et al., 2011). In addition, the potencies itself were also comparable between both assays. A range of small molecule antagonists tested on CCR2-overexpressing U2OS cells were overall equally potent in blocking CCL2-induced changes in CEI assays, as they were in blocking [^{35}S]GTPɣS binding and β-arrestin recruitment (Zweemer et al., 2013). Similarly, no differences in potency between CEI, a cAMP assay and a calcium mobilization assay were found when stimulating Free Fatty Acid receptor 1 (FFA1) overexpressing 1321N1 cells with either TUG-469 or GW9508 (Urban et al., 2013). Also, potencies for IL-8 stimulation of endogenous chemokine receptors CXCR1 and CXCR2 in primary human neutrophils were similar to previously reported values obtained from a chemotaxis assay (Verdonk et al., 2006). Hillger and colleagues showed comparable potencies for JWH133 on Cannabinoid 2 receptor (CB2) endogenously expressed on patient-derived lymphoblastoid cell lines (LCLs) between CEI and a conventional cAMP assay (Hillger et al., 2015). For a variety of noncompetitive antagonists of metabotropic glutamate receptor 1 (mGLUR1), similar rank order potencies were observed between the CEI and calcium assay using stably transfected CHO cells (Scandroglio et al., 2010). At the same time, the potencies obtained for a panel of agonists were also similar for both assays.

Data obtained with conventional drug discovery- and CEI assays are, however, not always fully comparable. Guo and colleagues stimulated HEK293 cells overexpressing a human Adenosine receptor A2a (hAA2R) with a variety of ligands and showed that the compound efficacies based on a cAMP assay and CEI assay correlated well. However, clear differences between both assays were found when comparing the compound potencies (Guo et al., 2012). Also, even though chemokines CXCL9, CXCL10 and CXCL11 were shown to have comparable agonistic potency on the chemokine receptor CXCR3 when tested using CEI, they clearly had distinct potencies in a cAMP reporter gene assay and a β-arrestin2 recruitment assay (Watts et al., 2012). Scandroglio and colleagues reported similar potencies for the small molecule JWH133 between the xCELLigence assay and the inhibition of forskolin-induced cAMP production when applied to cannaboid receptor 2 (CB2) transfected CHO cells (Scandroglio et al., 2010). CP55940, however, was more potent in inducing a CEI response than in inhibiting forskolin-induced cAMP production. Moreover, when a range of complement component 5a receptor 1 (C5aR) compounds were tested on CHO−C5aR cells and Human Monocyte Derived Macrophage cells (HMDM), lower potencies were obtained from the xCELLigence assay in comparison to an ERK - and binding assay (Halai et al., 2012). In a similar way, CXCL12 had a lower potency on U87.CD4.CXCR4 cells for CEI (Doijen et al., 2017) when compared to the calcium mobilization assay performed on the same cells (Van Hout et al., 2017). Lower potencies for a range of Protease Activated Receptor 2 (PAR2) agonists on 16HBE14o cells were also found using CEI measurements in comparison to the calcium mobilization assay performed on the same cells (Boitano et al., 2011).

2.4. CEI to evaluate biased signaling

Depending on the nature of the ligand or the cellular environment in which GPCRs are expressed, different signaling pathways may be activated (Kenakin, 2011). Biased activity of ligands can be overlooked by assays that focus on a single readout as compounds with limited efficacy or potency for stimulating or blocking a given pathway, might still potently activate or inhibit another branch downstream of that same receptor. To identify biased compounds, multiple assays must currently be combined, and the relative activity of the compounds in each assay needs to be compared properly. This is labour intensive, time-consuming and not always a trivial task.

Exploiting this signaling bias in drug discovery might lead to compounds that act on “therapeutic pathways” while minimally disturbing other receptor-mediated events (Violin et al., 2014). Such biased compounds could help counteract side effects or lower drug tolerance, and thus might become important pharmacological tools for chronic dosing of *e.g.* opioid analgesics (Schmid et al., 2017). Conceptually, CEI measurements should allow the detection of biased compounds due to the integrated nature of the response that is detected. Despite this potential advantage, only few reports have shed some light on this so far.

2.4.1. G protein-dependent vs G protein-independent pathways

The clearest example of GPCR signaling bias comes from ligand-directed differential engagement of G proteins versus β-arrestin molecules (Rajagopal et al., 2010). For example, the three natural CXCR3 ligands (the chemokines CXCL9, CXCL10 and CXCL11) display different potencies in a β-arrestin2 recruitment assay (Watts et al., 2012). Whereas CXCL11 is a full agonist and CXCL10 a partial agonist for β-arrestin2 recruitment, CXCL9 does not recruit β-arrestin2 at all. CXCL9 is also the least potent in CXCR3 binding and in inhibiting forskolin-induced cAMP production via G_i-activation, while CXCL11 was the most potent in these assays. Hence, CXCL9 can be considered as a biased ligand for CXCR3, only inducing G_i signaling without β-Arrestin signaling (Watts et al., 2012). All three ligands induced CEI profiles that reached a peak (after 8 min) with a shoulder (after 15–20 min). The profile for VUF10661, a synthetic CXCR3 agonist, also a reached a peak but without shoulder. Interestingly, CXCL9 induced higher maximal CI values than similar concentrations of CXCL10 and CXCL11 even though it was least potent in inhibiting forskolin-induced cAMP production via G_i-activation. This might be explained by CXCL9's inability to recruit β-arrestin2 molecules that are known to desensitize G protein-dependent signaling and lead to receptor internalization (Watts et al., 2012). The CI decreased after the peak and shoulder was reached, but for none of the ligands the value returned to baseline CI. CXCL9, the ligand that is unable to induce β-arrestin2 recruitment, stabilized at a higher CI value than CXCL10 and CXCL11 while VUF10661, a superagonist for β-arrestin2 recruitment, stabilized more quickly than the other ligands (30 min after ligand addition) (Watts et al., 2012). This implies that increased β-arrestin recruitment can influence the kinetics of the CI response. Whether the changes in kinetics are due to desensitization or other β-arrestin related processes should be further investigated. Because β-arrestin-mediated GPCR internalization often occurs in an actin cytoskeleton–dependent manner (Krilov et al., 2008; Laroche et al., 2005; Scott and Peters, 2010), it may be possible that CEI measurements could be influenced by receptor internalization/desensitization in a more direct manner as well.

Since the CXCR3 CEI profile, amongst other G_i-coupled receptor

profiles (Doijen et al., 2017; Hillger et al., 2015; Peters and Scott, 2009), could be completely blocked by pre-incubation with Pertussis toxin (PTX), a toxin that catalyzes ADP-ribosylation of G_i proteins (Locht and Antoine, 1995), the receptor's CEI profile seems to result solely form G protein-dependent signaling events (Doijen et al., 2017; Watts et al., 2012). Recent data obtained with cells overexpressing CXCR7, a chemokine receptor devoid of G protein-signaling (Balabanian et al., 2005; Burns et al., 2006; Thelen and Thelen, 2008), but capable of β-arrestin recruitment, ERK activation and internalization (Balabanian et al., 2005; Décaillot et al., 2011; Rajagopal et al., 2010) are in good agreement with this view: When challenged with CXCL11 or CXCL12, both natural chemokine ligands for CXCR7, no changes in CEI could be detected in CXCR7 overexpressing U87-MG cells (Doijen et al., 2017), while ERK activation was detectable via western blotting (personal observation). The lack of response from receptors that activate G_i proteins in the presence of PTX together with the absence of a response from a receptor that only recruits β-arrestins, suggests that β-arrestin recruitment and signaling by itself does not yield a CEI response. However, as previously suggested, other β-arrestin mediated processes might influence the CI response kinetics of G protein-dependent signaling. Nevertheless, Kamermann and colleagues suggested, using a variety of biased GPR109A agonists, that a transient negative response observed within the first 5 min after ligand addition correlates with β-arrestin-dependent signaling (Kammermann et al., 2011). Also here the response stabilization after reaching the CI peak value is faster for ligands with strong β-arrestin activation compared to ligands with some and ligands with no β-arrestin activation.

2.4.2. Cell type dependency of CEI responses

As many GPCRs couple to multiple G_α proteins, GPCR ligands can also be biased in terms of the G protein-signaling cascades. As such, blocking specific G proteins could be an interesting therapeutic approach in which one could 'bias' GPCR signals by inhibiting only a subset of these signals (Campbell and Smrcka, 2018).

Between different cell types 'apparent' bias can exist due to differences in the relative abundances of G_α proteins or other pathway components. One study showed that dopamine D1/D5 receptor stimulation by dopamine resulted in clearly distinct impedance profiles in U-2 Osteosarcoma cells (U-2 OS) compared to neuroepithelioma cells (SK-N-MC). In the former cell type, the D1/D5 receptor might be more G_s-coupled while in SK-N-MC cells it might be more $G_{i/o}$-coupled (Peters and Scott, 2009). By comparing the response of a variety of C5aR ligands on CHO-C5aR cells overexpressing the receptor with endogenous receptor responses on HMDM, differences in impedance profiles were observed between the two cell types, with a more complex pattern for cells endogenously expressing the receptor (Halai et al., 2012). This is not entirely surprising as endogenous receptor signaling versus signaling in receptor overexpression systems can lead to differences in G protein-coupling (Shapira et al., 2000) and even in binding affinity (Watson et al., 2000). The latter could result from conformational changes caused by the difference in coupling. In addition, possible cell type-dependent differences in receptor heterodimerization can contribute to signaling differences (Prinster et al., 2005). The radically different β2-adrenergic receptor (β2AR or ADRB2) CEI profile elicited by isoprotenerol in transfected HEK293S – versus Vascular Smooth Muscle Cells (VSMC) could be explained in a similar manner as the receptor can couple to both G_s and G_i (Stallaert et al., 2012). The CEI response of the Cannabinoid receptor 1 (CB1) to CP55490 and WIN55212-2 also differs when evaluated in CB1 transfected HEK293 versus CHO-k1 cells. As CB1 can activate both G_s and G_i, it is again possible that different G protein-signaling events dominate in the different cell types (Peters and Scott, 2009). This was confirmed using pharmacological agents. Upon preincubation with PTX Peters and Scott observed a CI response shift from positive to negative for stimulated CB1 expressing CHO-k1 while for stimulated CB1 expressing HEK293 cells only the negative phase remained upon PTX preincubation (Peters and Scott, 2009). Note that differences in CEI profile of the same GPCR between different cell lines are not necessarily due to differences in G coupling but could also be caused by other downstream pathway components.

2.4.3. CEI pathway deconvolution

One of the challenges of CEI is the fact that it's a "black box" system meaning that the kinetic profile is not necessarily attributed to one particular pathway. To determine the molecular components that contribute to the overall kinetic profile, the profiles can be deconvoluted using pharmacological inhibitors or activators. Wegener et al. (1999) were able to mimick the G_s-coupled β2-adrenergic receptor response in BAECs using forskolin, 8-(4-chlorophenylthio)-cAMP and 3-isobutyl-1-methylxanthine as these were able to activate the same pathway (Wegener et al., 1999). Stallaert and colleagues blocked G_s-, G_i- and $G_{\beta\gamma}$-signaling and other β2-adrenergic signaling events with pharmacological inhibitors in HEK293-ADRB2 cells and determined the contribution of each pathway to the overall CEI response (Stallaert et al., 2012). In this manner, they discovered a new, previously undescribed, β2-adrenergic-promoted Ca^{2+}-mobilization event. The CEI profiles mediated by the chemokine receptors CXCR3 and CXCR4 have been studied in detail as well (Doijen et al., 2017; Watts et al., 2012). Activation of both receptors by their natural ligands results in a similar CEI response, which is predominantly mediated by the G_i-protein since it can be fully blocked by pre-incubating with PTX. A similar positive, transient and PTX-sensitive CEI response can be observed when stimulating the G_i-coupled CB2 receptor with JWH133 in LCLs (Hillger et al., 2015). CXCR4 activation by its natural agonist CXCL12 also triggers intracellular Ca^{2+}-release. The G_q-inhibitor YM254890 totally blocks the CXCL12-induced release of Ca^{2+} in a Ca^{2+}-mobilization assay, while it only moderately impacted the CXCR4 CEI profile (Doijen et al., 2017). This finding suggests that CXCR4-induced calcium signaling does not strongly contribute to the CEI response and that other downstream targets are important for induction of CXCR4-related CEI changes. Similarly, Scandroglio and colleagues reported that BAY36-7620, an antagonist that totally blocks calcium mobilization downstream of the glutamate receptor mGluR1, did not affect the CEI profile upon receptor activation (Scandroglio et al., 2010). Meshki and colleagues also reported that blocking the Neurokinin 1 receptor receptor-induced calcium response with the compound U73122 in transfected HEK293 cells did not affect the CEI profile (Meshki et al., 2009). Whether this calcium-signaling independence of the CEI response is limited to some receptors or cell lines, or can be generalized to many receptors remains unclear.

ERK1/2 activation via CXCR7 did not elicit a CEI response (Doijen et al., 2017). However, Thirkettle-Watts and colleagues demonstrated, using various pharmacological blockers, that Akt and ERK1/2 phoshorylation contributed to independent features in the morphine-induced CEI profile of the μ-opiod receptor (MOR) transfected in CHO cells. The initial major peak was largely influenced by Akt phosphorylation whereas the secondary peak strongly depended on ERK1/2 phosphorylation (Thirkettle-Watts, 2016). These two, possibly independent, events described as a response peak and a shoulder are often observed for G_i-coupled CEI profiles and become more apparent at certain concentrations. The ability to dissect different signaling events using CEI in combination with pharmacological blockers demonstrates the potential of CEI for basic GPCR research while also it could facilitate the discovery of biased drug candidates. Detecting multiple signaling events at once can be a disadvantage as well because two separate, simultaneously activated pathways with opposing biological effects could cancel each other out. In other words, this could lead to a false negative CEI response outcome.

Fig. 1B gives a schematic overview of the different GPCR-induced pathways that could contribute to the CEI response. Fig. 1C and D shows characteristic impedance traces resulting from activation of the different G_α proteins. These traces are generalizations and are inferred

from the response patterns in Table S1. Note that the figure is a simplification and that the depicted processes and their effects on the impedance might not be representative for all GPCRs or cell types.

2.5. Discerning different types of GPCR-targeting compounds

GPCR drugs might either stimulate GPCR activity as (partial) agonists or biased agonist, or prevent receptor activation by its natural ligand(s) (*i.e.*, receptor antagonists). Inverse agonists are capable of reducing the basal or constitutive receptor activity (Wacker et al., 2017). More recently, allosteric modulators of GPCR activity emerged as a class of receptor-modifying agents with potential benefits over classical agonists and antagonists (Wacker et al., 2017). Recently, the number of biased agonists and allosteric modulators has increased in clinical testing as these are increasingly considered to have favorable properties for manipulating GPCRs (Hauser et al., 2017).

Various reports demonstrate that CEI allows identification and characterization of different types of GPCR-targeting compounds (*i.e.*, agonists, antagonists, inverse agonists, allosteric modulators) in one and the same assay (Chen et al., 2015; Doornbos et al., 2018a; Meguro et al., 2018; Moreno Delgado et al., 2017; Peters et al., 2007; Scott and Peters, 2010; Tagami et al., 2016; Zweemer et al., 2013) and this in a more relevant setting (at 37 °C on live cells and in culture medium) than many of the traditional assays.

For instance, Chen and colleagues demonstrated that the acetylcholine-induced CEI response on Muscarine receptor 4 could be potentiated by the positive allosteric modulator (PAM) LY2033298 and this potentiation was similar to the cooperativity estimated for ERK1/2 phosphorylation, while being higher than the cooperativity for inhibition of forskolin-induced cAMP accumulation (Chen et al., 2015). In a study by Meguro et al. (2018), the neuropeptide oxytocin (OT) was shown to enhance MOR signaling of various opioids as PAM. Even though no changes in affinity or potency were measured when OT was present, its presence resulted in a clear increase in efficacy measured using CEI (Meguro et al., 2018).

Doornbos et al. reported opposite changes in the impedance for ligands with opposite pharmacological effects on the metabotropic glutamate receptor 2 (mGLUR2). Both the agonist LY354740 and the PAM JNJ-46281222 increased the impedance while antagonist LY341495 and a negative allosteric modulator (NAM) RO4491533 decreased the impedance of a consitutively active mGLUR2 in CHO-k1 cells (Doornbos et al., 2018a). The increase in impedance induced by LY354740, typically seen for G_i-coupled receptors, as well as the decrease in impedance elicited by LY341495, could be blocked by PTX, suggesting that both processes are G_i-mediated. As preincubation with LY341495 resulted in more pronounced positive LY354740-induced CEI profiles while preincubation with the NAM did decrease the LY354740 response, they speculated that LY341495 in fact behaves as an inverse agonist instead of an antagonist. This inverse agonism was further confirmed using PAM and NAM pretreatment experiments (Doornbos et al., 2018a). The ability of CEI to discern ligands with various activities is a big advantage of the technology (Doornbos and Heitman, 2019).

2.6. The link between residence time and CEI profile

Using CEI, Nederpelt et al., (2016) demonstrated that slower dissociation from NK1 of tachykinins and derivatives thereof on U-251 MG cells correlated with increased efficacy (Nederpelt et al., 2016). Differences in association rate did correlate with ligand potencies but did not influence the CEI response shape. A similar positive correlation between potency of orthosteric ligands and association rates was observed by Doornbos et al. for mGLUR2 on mGLUR2 expressing CHO-k1 (Doornbos et al., 2018b). Interestingly, glutamate's dissociation rate from mGLUR2 was decreased in the presence of the PAM JNJ-46281222. This led to increased affinity of glutamate for the receptor and enhanced glutamate potency on mGLUR2 expressing CHO-k1 cells in a CEI assay. This change in dissociation also lengthened the glutamate-induced CEI response. CEI profiles seem to be influenced by differences in dissociation rate and thus could be a valuable method to assess the effect of residence time on the efficacy of GPCR ligands, which could be important for improved *in vivo* efficacy (Doornbos et al., 2018b).

2.7. Inclusion of patient-derived cell samples for precision medicine

In contrast to many conventional GPCR assays, CEI technology is sensitive enough to measure receptor activity in native environments *e.g.,* in cancer cell lines, primary cultured cells and patient-derived cell samples. By applying a large variety of ligands, McGuinness describes the recording of impedance responses from a broad set of GPCRs endogenously expressed in human primary prostate and stromal cells using the CellKey system. This form of receptor profiling is named receptor panning and gives an indication about the active endogenously expressed GPCRs (McGuinness, 2007). After receptor panning, the presence of a particular GPCR on the cell surface can be validated pharmacologically using appropriate agonists and antagonists. An overview of the established and primary cell types used for receptor panning experiments is given in Table S1.

Monitoring drug responses in patient-derived cell samples can be highly informative and can significantly contribute to personalized medicine (Kodack et al., 2017). Hillger and colleagues demonstrated that activation of the cannabinoid receptor 2 (CB2R) in patient-derived lymphoblastoid cell lines (LCLs) resulted in clear CEI traces (Hillger et al., 2015). Later on they demonstrated that CEI measurements can detect differential CB2R drug responses in LCLs from a cohort of patients (Hillger et al., 2017). Of interest, the observed differences in drug response could be linked to the genetic variation in the GPCR that was targeted by the therapy (Hillger et al., 2017). LCLs from different patients responded differently to synthetic CB2R (partial) agonists. Based on the efficacies, the synthetic agonist WIN55212-2 behaved as partial agonist in comparison with JWH133 on two LCL cell lines and as a full agonist on others. The CEI changes elicited by the non-classical cannaboid CP55940 were most sensitive to the genotype while the CEI response to aminoalkylindoles (*e.g.* WIN552122-2) showed the least variation between individuals (Hillger et al., 2017). Besides differences in compliance or pharmacokinetics, genetic variation could help explain why so-called 'blockbuster' drugs only work in a subset of all patients (Hauser et al., 2018). CEI measurements may therefore be well-suited to identify whether a compound is fit for the general population or is prone to genetic differences (Hillger et al., 2017).

2.8. Evaluating off-target effects and cellular toxicity

Many drugs that target kinases or other proteins display activity on GPCRs. Such cross-reactivities can be predicted based on ligand similarities using chemo-informatics and can be confirmed later on experimentally (Lin et al., 2013). To detect such cross-reactivies early on in drug discovery, receptorome screens can be performed using a variety of assays such as a calcium mobilization assay or a radioligand binding assays. An overview is given by Strachan and colleagues (Strachan et al., 2006). Combining a label-free CEI assay with GPCR pathway blockers might be a more effective way to confirm or even help detect such cross-reactivities as the integrated readout covers multiple GPCR-activated pathways.

It is well established that CEI can be used to monitor cellular toxicity of compounds in real-time (Menotti et al., 2017). The results are highly comparable with other methods, but the real-time aspect can help determine the optimal time points to perform more specific endpoint assays (Ke et al., 2011; Xing et al., 2005). Moreover, it was shown that different types of compounds, including antimotitics, DNA damaging agents, and protein synthesis inhibitors, result in distinguishable

impedance profiles (Abassi et al., 2009). Hence, CEI can be used to detect deleterious effects of potential therapeutic agents that are meant to target GPCRs. Very subtle aspecific effects can be detected, as long as they result in changes in the cell's morphology. Since the CEI assay for GPCR studies is harmless to cells, information on toxicity of the candidate drugs can be gained by measuring the effect of the compound over a longer time period in addition to measuring its effect on GPCR activity without additional costs. CEI measurements might therefore be a preferred way to detect aspecific effects of compounds early in drug discovery, especially when assayed in physiologically relevant cell types.

2.9. Where does CEI fit in the GPCR drug discovery pipeline?

The low (8-well) to middle (96-well) throughput of most available CEI systems limits its use for initial compound screening of extensive compound libraries. Nevertheless, it could be used for smaller more specialized libraries. Also, with the recent introduction of a 384-well device, ACEA biosciences does enter the market for HTS as the system can be implemented in fully-automated screening workflows.

The technology offers some advantages for initial screening of compound libraries: The integrated readout allows for a screening that is less “biased” compared to many other assays that are currently being used such as the recording of second messenger levels. The CEI assay is also applicable to most GPCR classes as signaling downstream all major G_a protein classes can be detected. Thus, when using this more generic readout it's not required to have prior knowledge on the activated pathways of the GPCR of interest.

In most cases, CEI will be useful during the H2L and lead optimization stages. As the CEI assay can be performed in a more physiologically relevant setting than most alternative cell-based assays, the results might be more transferable. CEI measurements could lead to more accurate potency prediction because they can be used with relevant cell types that are usually better at resembling *in vivo* potencies than overexpression systems. In addition, the integrated nature of CEI responses will help give a more overall prediction of the potency since it does not only rely on one single pathway. Relevant cell types include patient derived samples with different genetic backgrounds which opens new avenues for personalized medicine as well. Due to the non-harmful readout of CEI, both GPCR activity and toxicity could be evaluated within the same assay without additional costs. Finally, discovery campaigns would benefit from ways to find more specialized compounds in order to decrease the possibility of adverse effects. In this regard, CEI was recently shown to be able to deliver detailed GPCR pharmacology of compounds with the ability to discern inverse agonists, antagonists and allosteric modulators.

3. Suggestions for further improvements and exploiting the full potential of CEI

As described above, a CEI assay has some clear advantages over traditional GPCR assays. However, the true potential of this technique has not yet been fully exploited. We suggest that improved and standardized data analysis and data modelling as well as the experimental dissection of the signaling pathways that contribute to the overall CEI response would constitute important, meaningful advances to exploit the full potential of this technique.

3.1. Data analysis

Straightforward analysis is desirable for a screening assay. For instance, one readout frequency can be chosen and the CEI value at one time point just prior to ligand addition can be compared to one after addition. In this manner, compounds can be detected that alter the impedance value with respect to a reference profile. Impedance devices such as the ECIS system allow monitoring the CEI at multiple frequencies and over multiple time points. For each frequency, impedance values prior to ligand addition can be compared to those at a certain time point after ligand addition to find the frequency at which the difference between the impedance values is most pronounced. This frequency can then be chosen to investigate the response over time. The optimal readout frequency may be dependent on the cell type and the application. However, since different impedance-based biosensors with different frequency settings are successfully used for GPCR research as well as for other applications, the choice of a particular frequency may in most cases not be that critical.

Impedance values measured across different frequencies and over a defined time interval can be represented with a limited number of interpretable parameters in terms of cellular properties using an equivalent circuit model (ECM). The ECIS platform's software can represent CEI data across multiple frequencies using an ECM. CEI response data over multiple frequencies can also be reduced using curve-fitting as demonstrated by Ciambrone and colleagues (Ciambrone et al., 2004). They used a form of complete and orthogonal polynomials to fit the data at each time point and plotted the obtained polynomial parameter values over time.

Typically, CEI responses for GPCR studies are measured at one or a limited number of frequencies. In addition, these responses are not usually thoroughly investigated. Instead the data is reduced; so only the response intensity or area under the curve values are used while very often response kinetics information is neglected. Future modelling efforts that help describe the kinetics of CEI ligand responses could be useful for GPCR drug discovery, especially if these models can provide biologically relevant information on subprocesses present in the integrated CEI response.

Besides preservation of kinetic information using modelling, clustering algorithms can be used to classify responses. For example, Stallaert and colleagues performed unsupervised clustering of the CEI changes elicited by a variety of β2AR ligands using visual assessment of a tendency method. This revealed that the β2AR ligand responses could be divided into 5 different classes, thereby providing a richer signaling repertoire for this receptor than previously recognized (Stallaert et al., 2012). Abassi and colleagues measured CEI responses of small molecule compounds, using a xCELLigence system after which they performed agglomerative hierarchical clustering (Abassi et al., 2009). They demonstrated that compounds with similar activities elicited similar CEI responses. The availability of large standardized data sets would greatly facilitate these types of analysis.

Identifying receptors likely activated by test compounds can be achieved by comparing their profiles with those of reference compounds known to selectively activate particular receptors. This is exemplified by a study where HMDMs were stimulated with C5a, C3a and a range of peptides. These peptides induced similar CEI responses as C3a suggesting they acted upon the same receptor. This was confirmed using small molecule inhibitors (Halai et al., 2014). Being able to make such predictions during a screen benefits further drug development, and the prediction can be facilitated and objectified by clustering algorithms.

3.2. Impact of frequency selection and electrode layout

The electrode layout, incorporated in the assay plate, can influence the sensitivity and reproducibility of CEI measurements. For example, the ECIS plates with small detection electrodes and a large reference electrodes are very sensitive and can detect cellular micromotion (Giaever and Keese, 1991) but at the same time are more prone to variation due to the small detection area. Nowadays, most available systems have plates with interdigitating electrodes (xCELLigence, ECIS, …). As a result, the covered detection area has increased and thus allows more cells to be monitored at once.

Different instruments often employ different data acquisition and data processing strategies. Therefore, it is not always clear which one is

best suited for which application. These differences impair the comparability of data.

The readout frequency can also influence the results. This is exemplified by Stolwijk and colleagues, showing how the histamine dose-response curve on Human Dermal Microvascular Endothelial Cells (HDMECs) clearly shifts when measuring the resistance (R) of the complex impedance at 40.000 Hz in comparison to 4000 or 400 Hz (Stolwijk et al., 2015). This shift was not observed when the magnitude |Z| of the complex impedance was plotted. Even though it is suggested by ECIS that barrier function of endothelial cells is best measured using resistance at 4000 Hz or lower, as at these frequencies the current will pass the cells mainly in a paracellular fashion, the xCELLigence system has also been used to successfully monitor barrier function (Sinha et al., 2016; Sun et al., 2012; Twiss et al., 2012) which suggests that the phenomenon can still be detected at higher frequencies.

3.3. Suggestions for improved data comparability

For membrane receptor research, we recommend, if possible, to scan initially the impedance profile at multiple frequencies. We suggest this because there is still a lot of uncertainty as to the optimal frequency, as it may depend on the cellular process one is interested in, the cell type and/or the receptor itself. In view of the increasing demand to provide open data, it may be useful to develop a data standard for CEI measurements. As such, if no particular frequency clearly stands out, and if a clear response is observed at 10 kHz, we suggest to use and report the impedance at 10 kHz. This frequency can be chosen on most commercially available devices and seems to be well-suited for GPCR research. In fact, even when chosing another frequency, it would be interesting to also include the response at 10 kHz, as it would improve result comparibility between independent research groups and between different instruments. In addition, it would facilitate the generation of large standardized response data sets. Making non-normalized CEI values publicly available would improve data comparability as well. In this manner, information on electrical properties of the studied cell type as well as information that might help explain differences in shape and intensity of CEI responses is conserved (Stolwijk et al., 2015).

3.4. The use of molecular approaches to aid studying CEI responses

Earlier on, we have discussed how pharmacological inhibitors can and have been used to study CEI responses. However, pharmacological inhibitors display limited selectivity at high concentrations and can provoke off-target effects, which in turn can hamper data interpretation. To better identifiy the signaling pathways that contribute to the CEI response, one could rely on molecular biology approaches. It might be interesting to manipulate cells using ectopic expression of complementary DNA (cDNA) encoding wild type or mutated protein, or by knocking down or knocking out specific proteins. In this respect, Artus and colleagues used siRNAs against PAR3 showing that the Wnt/Par/aPKC PCP pathway, which is important for tight junction reassembly and regulating blood brain barrier properties, significantly contributed to the CEI response (Artus et al., 2013). Another study used siRNAs to demonstrate that the observed CEI profile was indeed mediated via LPAR6 (Yanagida et al., 2009). However, the use of siRNAs has its limitations as well. Effective silencing is sometimes difficult to achieve as it depends on sequence and structure of the siRNA as well as on the cell type's receptiveness to siRNA uptake and the half life of the target protein. Moreover, siRNAs can have nonspecific effects *i.e.* effects on the cell beyond those caused by gene silencing (Sledz and Williams, 2005). In general, studies focused on unraveling CEI profiles could benefit from implementing molecular biology approaches besides using pharmacological inhibitors. Specifically, the use of G_{α} protein knock outs and knockouts of β-arrestin molecules will vastly improve our understanding of the integrated CEI profiles elicted by GPCR activation.

4. Conclusions

CEI is a valuable assay for early use in drug discovery, as it can provide additional insights on ligand activity and bias. In comparison with the limited dynamic resolution of most label-based assays, CEI measurements have high temporal resolution allowing real-time kinetic measurements of receptor-mediated signaling. In contrast to many label-based assays, the technology is harmless which means that GPCR activity and toxicity could be evaluated within the same assay without additional costs. Hence, it is an excellent method to assess the quality and specificity of compounds early on in drug discovery. A screening with CEI will be less “biased” than conventional screening as the readout is not focused on one pathway. The CEI assay can be used for initial screening, especially of smaller compound libraries, on well-known targets as well as on understudied targets for which downstream signaling is still largely unknown. Because screening using CEI can be done on more physiologically relevant cell types and often in a more physiologically relevant setting, the results might be more transferable (*e.g.* better potency predicition) than with conventional GPCR assays. These features make CEI especially attractive for H2L and lead optimization as well as for fundamental GPCR research. Implementing CEI technology early on in drug discovery might therefore help reduce the compound drop-out later on in the pipeline, due to more thorough testing during the *in vitro* stage.

However, CEI lacks intracellular spatial resolution, which is required to resolve many important cellular processes such as intracellular trafficking as well as location and organization of signaling molecules. As such, side-by-side comparison with other GPCR assays will remain crucial. Moreover, some intracellular molecular events, which could be key for pursuing certain therapeutic strategies, do not always seem to contribute to the CEI signal. These include G protein-independent events and sometimes calcium-related events. Thus, the CEI assay is complementary to conventional GPCR screening assays, but cannot necessarily replace them.

Standardized protocols for CEI measurements are desirable to improve data comparability between independent research groups. Moreover, if raw electrical impedance data would be made publicly available, such efforts could accelerate. In addition, further data analysis and the use of molecular approaches will be required in order to fully exploit the technological potential and the integrated readout.

Declaration of interests

- The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
- The authors declare the following financial interests/personal relationships which may be considered as potential competing interests

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Appendix A. Supplementary data

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