Label-Free Whole Cell Biosensing for High-Throughput Discovery of Activators and Inhibitors Targeting G Protein-Activated Inwardly Rectifying Potassium Channels

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Supporting Information

ABSTRACT: Dynamic mass redistribution (DMR) and cellular dielectric spectroscopy (CDS) are label-free biosensor technologies that capture real-time integrated cellular responses upon exposure to extra- and intracellular stimuli. They register signaling routes that are accompanied by cell shape changes and/or molecular movement of cells proximal to the biosensor to which they are attached. Here, we report the unexpected observation that robust DMR and CDS signatures are also elicited upon direct stimulation of G protein-activated inwardly rectifying potassium (GIRK) channels, which are involved in the regulation of excitability in the heart and brain. Using ML297, a small-molecule GIRK activator, along with channel blockers and cytoskeletal network inhibitors, we found that GIRK activation exerts its effects on cell shape by a mechanism which depends on actin but not the microtubule network. Because label-free real-time biosensing (i) quantitatively determines concentration dependency of GIRK activators, (ii) accurately assesses the impact of GIRK channel blockers, (iii) is high throughput-compatible, and (iv) visualizes previously unknown cellular consequences downstream of direct GIRK activation, we do not only provide a novel experimental strategy for identification of GIRK ligands but also an entirely new angle to probe GIRK (ligand) biology. We envision that DMR and CDS may add to the repertoire of technologies for systematic exploitation of ion channel function and, in turn, to the identification of novel GIRK ligands in order to treat cardiovascular and neurological disorders.

INTRODUCTION

As G protein-gated inwardly rectifying potassium (GIRK, Kir3) channels are implicated in an increasing number of pathologies, they are gaining focus as targets for pharmacological intervention. 1,2 They exist as hetero- or homotetrameric structures comprised of one or more of four subunits (GIRK1-4), depending on tissue distribution. 3,4 GIRCs are activated by G/βγ subunits of stimulated Gi protein-coupled receptors, thereby inducing neurons and cardiac pacemaker cells to hyperpolarize and, as such, regulate cellular excitability within heart and brain. GIRK channels have also been linked to pathologies related to perturbations of rhythmic action potential firing, such as epilepsy, cardiac arrhythmias, and Alzheimer’s disease among others. 5,3−6 In order to combat these diseases, unrelenting search for new pharmacological treatment options has been underway. The recent development of the small molecule GIRK activator ML297 is one such example that has shown positive effects in preclinical models of epilepsy, 7 anxiety, 8 and Alzheimer’s disease. 9 Yet, identification of new GIRK channel agonists with subtype selectivity and tissue specificity is still an ongoing endeavor in search for molecules with therapeutic potential.

Currently available methods for the investigation of ion channels and their ligands include automated electrophysiol-
ogy and ion-specific fluorescence dyes. The former requires electrical access to the cell interior by either sealing a microelectrode onto the cell surface with gigaohm resistance (classical patch clamp) or dislodging the cells from their substrate in order to perform automated measurements (automated patch clamp). The latter relies on dyes which are loaded onto and trapped within cells, where they react sensitively to influx of specific ions or changes in potential. Both techniques, with patch clamping still considered as gold standard, are powerful, yet technically challenging, time consuming, and only offer an insight into a channel’s conducting function and upstream regulatory elements.

Optical-based dynamic mass redistribution (DMR) and cellular dielectric spectroscopy (CDS) are label-free biosensor platforms. They are well established for the detection of integrated responses in real-time when living cells are exposed to pharmacologically active stimuli. Rather than relying on specific endpoints, such as changes in electrical potentials or accumulation of ions, both biosensors deliver more complex time-resolved activity profiles of entire cells, without the need for physical access to cells or artificial labels (Figure 1a,b). In addition to their original purpose of visualizing activity of signaling-competent proteins within living cells, we here show that DMR and CDS also serve to monitor the cellular consequences that occur upon direct GIRK channel activation. We present the molecular underpinnings associated with GIRK-mediated cell shape changes and raise the possibility that this mode of activation may be mechanistically distinct from the endogenous Gαi-βγ activation pathway. Thereby, our results do not only present a novel method for detection of previously unrecognized GIRK-mediated downstream effects but also have important implications for GIRK ligand drug discovery.

**RESULTS AND DISCUSSION**

**GIRK Activation Elicits Specific Label-Free Responses.**

Label-free methods are neither widely used nor considered appropriate to investigate ion channel behavior because the movement of ions per se through the plasmalemma is not believed to register as mass movement. On the other hand, evidence is available to suggest that selected ion channels can directly influence cellular signaling mechanisms through...
processes that are not directly linked to their ion flux function.\textsuperscript{29,30} We were intrigued to elicit robust label-free signatures upon stimulation of GIRK1/2, the neuronal GIRK channel prototype, with ML297, a small molecule GIRK activator selective for GIRK1-containing channels.\textsuperscript{8} Thus far, the GIRK subclass of inwardly rectifying potassium channels has been linked to "conducting functions" related to ion flow but not to "nonconducting functions" related to cellular signaling mechanisms.\textsuperscript{1,4,7,8,31} Therefore, we set out to investigate the molecular basis underlying GIRK channel activation-induced label-free whole cell responses. In HEK cells recombinantly expressing the functional GIRK1/2 subunit combination, we found that treatment with ML297 evoked concentration-dependent DMR response profiles that were characterized by an initial transient negative phase followed by a rapid ascending phase (Figure 1c,d). No changes in DMR were observed when native HEK cells were exposed to ML297, confirming the GIRK-specific origin of mass movement.\textsuperscript{15,16,18,25,26} Therein, activation of GIRK1/2 with ML297 led to characteristic noninverting and concentration-dependent negative signals with similar steady kinetics over 3600 s in a strictly GIRK-dependent manner (Figure 1f,g).

Control stimulus ATP was equally effective in both cell lines again confirming that ML297 recordings were no consequence of generally enhanced responsiveness in GIRK-expressing cells (Figure 1b). Notably, both methods revealed similar pEC50 values (Table S1), 5.45 and 5.50 for DMR and CDS, respectively, which is an order of magnitude lower than those observed in patch clamp\textsuperscript{8} and thallium flux\textsuperscript{32} readouts.

To further corroborate that the label-free records specifically originated from GIRK1/2 channel activation, and to investigate whether mass movement is functionally linked to ion flux, we pretreated GIRK1/2 expressing cells with three distinct channel blockers: (i) the small molecule SCH23390 (SCH), initially described as dopamine receptor agonist but known to also block GIRK currents,\textsuperscript{33} (ii) Tertiapin Q (TPNQ), the stable form of the bee toxin peptide, which inhibits channel function via interaction with the base of the external pore,\textsuperscript{34,35} and (iii) extracellular barium (Ba\textsuperscript{2+}), which effectively blocks the channel pore.\textsuperscript{36} Incubation of cells with increasing concentrations of SCH (Figure 2a,b) and TPNQ (Figure 2c,d) dampened the ML297-derived signal in a concentration-dependent manner without shifting pEC50 values (Table S2), compatible with noncompetitive antagonism. Ba\textsuperscript{2+} only partially blocked the ML297-derived signal.
even at maximal concentration (Figure 2e,f), reminiscent of findings previously reported in electrophysiological recordings. Inhibitor effects were specifically mediated via GIRK1/2 channels because cell-intrinsic viability controls prostaglandin E1 (PGE1) and ATP were unaltered in inhibitor pretreated cells (Figures 2g−i and S1). Equivalent findings were obtained when GIRK1/2 cells were pretreated with SCH and TPNQ using bioimpedance measurements (Figure 3a−d). These results show that both label-free platforms display specific ML297-mediated cell responses that are impaired (Ba2+) or absent (SCH and TPNQ) in the presence of GIRK blockers in striking resemblance to results obtained with electrophysiological or ion-specific fluorescence dye assays. We conclude that label-free DMR and CDS portray the cellular consequences of GIRK channel opening and thereby reveal an apparent molecular link between ion flow and cell morphology changes, the molecular basis of DMR and CDS detection. Indeed, images extracted from time-lapse videos of living HEK GIRK1/2 cells revealed profound flattening of cells in a strictly ML297-specific manner consistent with the

![Figure 3](image1.png)

Figure 3. GIRK channel inhibitors suppress the impedance response to ML297. (a) Impedance profiles of ML297-activated GIRK1/2 in HEK cells pretreated with buffer, SCH, and TPNQ. (b) Concentration−effect curves of impedance AUC over 3600 s derived from traces in (a) (n = 3), pEC50 value of buffer-treated cells was determined as 5.29 ± 0.10. (c) Representative impedance traces in response to Cch (100 μM) and ATP (100 μM) in the presence of GIRK inhibitors. (d) Quantification of impedance records derived from (c) (n = 3).

![Figure 4](image2.png)

Figure 4. Time-lapse imaging of living HEK GIRK1/2 cells reveals ML297-mediated cell morphology changes. Representative bright-field images of HEK cells stably expressing GIRK1/2 at 0, 1, 2, and 3 h after treatment with buffer (a), 10 μM ML297 (b), or pretreated with 10 μM SCH prior to ML297 (10 μM) application (c). Changes in cell morphology from oval to flat shape are marked by arrows. One representative out of three biological replicates is shown. Scale bar, 50 μm, applied to all.
movement of mass towards the biosensor that was detected by DMR (Figure 4a,b). This motion was not observed when cells were pretreated with SCH (Figure 4c) and was absent in cells lacking the GIRK1/2 channel (Figure S2, for full time-lapse videos see the Supporting Information).

**Label-Free Readouts of GIRK1/4 Heterotetramers Display Inhibitor-Sensitive Responses to ML297.** Patch clamp and thallium flux assays report ML297 activity only if functional channel complexes bear the GIRK1 subunit.8,31 To interrogate whether the same molecular requirements apply to label-free whole cell biosensing, we took advantage of previously described functional and nonfunctional ML297-sensitive and nonsensitive GIRK subunit combinations.8,31 We obtained ML297-mediated and SCH-sensitive DMR recordings in HEK cells stably expressing GIRK1/4, the prototypical isoform of the cardiovascular system42 (Figure 5a). Cells expressing GIRK1 which contains the ML297 binding site but does not form functional homomultimeric channels (Figure 5b), or GIRK2, which forms functional channels but lacks the relevant ML297 binding site3,4,8 (Figure 5c) failed to produce any change in mass distribution readouts. Comparable results were obtained in CDS recordings where ML297-dependent SCH-sensitive traces were evoked only in GIRK1/4 but not in GIRK1 or GIRK2 expressing cells (Figure 5d–f). The observation that both readouts only detect ML297-mediated GIRK activation upon its binding to functional GIRK1 bearing channels confirms this molecular prerequisite to be shared for both label-free and electrophysiological measurements.

ML297-mediated and SCH-sensitive whole cell responses were also evoked in HL-1 cells, an immortalized murine cardiac cell line that endogenously expresses GIRK1/4 channels.43,44 More specifically, we observed concentration-dependent DMR profiles in response to ML297 reminiscent of those observed in the HEK background (Figure 6a). ML297 (Figure 6b) but not control stimuli (Figure 6c,d) were blunted by SCH pretreatment, suggesting GIRK-specific inhibition of cellular responses. We also noted enhanced fluctuations within DMR profiles in all SCH-pretreated samples (Figure 6b,d) which likely reflect the increase in spontaneous beating because of endogenous GIRK1/4 inhibition by SCH. From these experiments, we concluded that GIRK channel-mediated cell morphology changes are not restricted to the HEK system but likely are of more general relevance for cells that express GIRK under native conditions.

**Screening of Structural and Functional Analogs of ML297.** If DMR and CDS biosensors faithfully recapitulate the cellular effects triggered by direct GIRK activation, we reasoned that label-free activity rankings of GIRK activators with different potencies should match the profile obtained in thallium flux or patch clamp assays. To this end, we characterized a small library of structural ML297 analogs, all of which were previously characterized in thallium assays and/or whole-cell patch clamp electrophysiology in GIRK1/2-HEK cells152 (Table S3). Both DMR and CDS recordings revealed specific responses to S51 which were sensitive to inhibitor
treatment and absent in native HEK cells (Figures 7 and S3).

Although DMR also detected weak but significant activation by 553 (Figure 7a-c), this compound was inactive in CDS measurements across all tested concentrations (Figure 7d-f). Efficacy of direct GIRK activators varied somewhat between the two platforms (compare Figure 7c with 7f), yet pEC50 calculation portrayed 551 as more potent than ML297 and 553 for both methods, and, moreover, classified all remaining analogs as inactive. Thus, overall potency rankings were retained in label-free assays but concentration-effect curves for all active GIRK ligands were again shifted to lower concentrations as compared with thallium flux assays.

From these results, we concluded (i) that DMR and CDS are competent to differentiate between true and false positives akin to methods routinely applied for GIRK ligand characterization,8,11,31 and (ii) corroborate existence of a molecular link between direct GIRK activation and cell morphology changes.

**Gi-βγ Does Not Visibly Contribute to Direct ML297-Induced GIRK Activation in DMR Detection.** A mechanism alternative to direct GIRK activation is used endogenously by excitable cells and relies on Gβγ subunits that are released upon stimulation of Gi-coupled G protein-coupled receptors (GPCRs). Because it is known from whole-cell and single-channel patch clamp experiments that GPCRs via Gβγ and direct GIRK stimuli activate GIRK channels in distinct manners,8 we wondered whether DMR would recapitulate these mechanistic differences. One such difference consists in the necessity of Gβγ itself, which is required for GPCR—but not ML297-mediated GIRK activation.8 We used cell lines coexpressing the GIRK1/2 subunits along with the Gi-sensitive OXE-receptor (OXE-R) and Gi/o inhibitor pertussis toxin (PTX) to block liberation of Gβγ from Gi-βγ heterotrimers. Indeed, ML297-mediated DMR responses and thallium flux in PTX pretreated cells were likewise unaffected or slightly diminished (Figure 8a,b). On the contrary, Gi-βγ-dependent thallium flux induced by 5-oxo-ETE stimulation of OXE-R was completely blunted by PTX, and additionally by the specific OXE-R blocker Gore27, confirming its receptor origin (Figure 8c). From these data, we concluded that ML297-mediated DMR traces in GIRK expressing cells do not rely on Gi-βγ akin to ML297-mediated GIRK currents in thallium flux assays,8 again suggesting mechanistic commonalities between direct ion flux assay techniques and the more distal DMR detection. Because GIRK channels are pharmaco-
logical targets to reduce activity of excitable cells in cardiological and neurological disorders, discovery of ionicotropic downstream consequences following their direct activation should be of considerable relevance to GIRK channel drug development.

**ML297-Induced DMR in GIRK1/2 Cells Requires an Intact Actin Cytoskeleton.** Changes registered with DMR or CDS biosensors are a consequence of altered morphology and/or cell motion that occurs when cells spread or “contract” on the surface of these biosensors. To investigate the cellular origin that accounts for the observed mass movements in response to ML297, we preincubated cells with actin or tubulin polymerization inhibitors prior to DMR detection. Because inhibition of actin- or tubulin-dependent cytoskeletal restructuring may lead to cell detachment and register as loss of mass, this experiment was recorded in suspension instead of the adherent mode and with sufficient time to allow for baseline equilibration before ML297 addition (Figure S4a−c).

Actin inhibitors cytochalasin B 46 and latrunculin A, 47,48 but not the microtubule inhibitor nocodazole 49 attenuated ML297-mediated DMR responses in a concentration-dependent manner (Figure 9a−c). Importantly, inhibitor-treated cells remained viable during DMR detection (Figure S4d) and responsive to external stimuli that do not require an intact actin cytoskeleton such as Ca2+ release from intracellular stores 50 (Figure S4e). From these results, we concluded that the ML297 signal portrayed by DMR originated from changes in actin network rearrangement downstream of ionicotropic channel activity rather than channel conductance in its own right. Thereby, holistic DMR and CDS detection uncovered a previously unknown link between direct GIRK activation and reorganization of the actin cytoskeleton. We speculate that optical and impedance-based biosensing may become more widely applied in the field of ion channel research for those channels that elicit cellular effects beyond the simple conduction of ions.

**CONCLUSIONS**

The major finding of the present study is centered on the discovery that optical and impedance-based biosensing of living cells sheds light on molecular properties of GIRK channels and their ligands that were previously not seen. Routinely, ion channel targeting drugs are characterized using methods that record direct ion flow with patch clamp being the gold standard because of its unrivalled precision and accuracy. 10 Our study advances the field of ion channel drug discovery in an important way: we identified label-free DMR and CDS as additional technology platforms competent to portray GIRK channel activity beyond the mere conducting function. Apparently, methods that record only ion flux per se provide an incomplete picture of the spectrum of pharmacological activities of GIRK modulators. Indeed, several channels are known to engage in channel–protein interactions with cytoplasmic signaling molecules beyond the simple transport of ions through the plasma membrane. 19,30,51 These would be in need of additional methodologies to portray their biological actions in a more comprehensive manner. Slack (KCNq1.1, KCNT1, SLO2.2), a sodium-activated K+ channel and member of the inward rectifying K+ channel family, is one such example. 30 On the other hand, K+ channels such as BK (KCNA1, SLO1) and slip (KCNq1.2, KCNT2, SLO2.1) do not produce DMR upon direct stimulation, indicating that label-free whole cell responses are no general consequence of increased K+ conductance. 23 Because BK and slip—upon direct activation—do not produce DMR, and slack triggers mass movement independent of ion flux, 30 GIRK channels stand out among voltage-sensitive K+ channels in that they convert direct activation into cell morphology changes, the basis of DMR and CDS detection.

One caveat deserves particular mention here: the majority of recordings were obtained in HEK cells which express GIRK subunits recombiantly. The striking correlation between DMR profiles (this study) and GIRK electrophysiology 8,31 in this very cellular background, however, strongly suggests that a similar mechanism may operate in primary cardiac or neuronal cells. Indeed, HEK cells have proven an ideal non-neuronal host for functional expression and electrophysiological study of GIRK channels in the past. 8,31 Therefore our data do not only extend the repertoire of pharmacological assays for characterization of GIRK channel-modifying drugs by label-free noninvasive whole-cell biosensing but also identify GIRKs as a particular subgroup within inwardly rectifying K+ channels that connect ion flux to cytoskeleton-dependent cellular mechanisms. G protein-independent, direct GIRK activation is still poorly understood. The effects observed for ML297 in DMR and CDS may therefore be an important step to deepen our understanding how other extrinsic and intrinsic factors like ethanol or cholesterol, which also bypass G protein-gating, 52−55 contribute to regulation of excitable cells. The ability to capture previously unseen cellular mechanisms surrounding GIRK activation in phenotypic live cell assays, therefore, offers an unprecedented approach toward both...
identification of new GIRK ligands and mechanistic understanding of their biological effects. Given that DMR and CDS are high throughput-compatible, we anticipate that both hold much promise in the preclinical discovery pipeline for development of ion channel targeting drugs that regulate ion flux and, additionally, are in need of technologies to portray their nonconducting functions.

**MATERIALS AND METHODS**

**Chemicals and Reagents.** Synthesis of structural analogs was described previously.7,32 The analogs are referred to by the last three digits of their Vanderbilt University Code. For the full length code, see Figure 7a. The OXE-R antagonist Gore27 was prepared following the synthetic route provided in ref 56 and detailed in the Supporting Information. The Thallos AM reagent was purchased from TEFLabs (Austin, TX, USA), and PTX was from Life Technologies (Darmstadt, GER). In all experiments, Hank’s balanced salt solution from Thermo Fisher (Darmstadt, GER) supplemented with 20 mM N-(2-hydroxyethyl)piperazine-N’-ethanesulfonic acid (pH 7.2) was used as assay buffer.

**Stable Cell Lines.** Generation of HEK293 (HEK) cell lines stably expressing GIRK1/2 or GIRK2 were described previously.7 HEK cells stably expressing GIRK1/2 channels and the Gi-sensitive 5-oxo-ETE receptor OXE-R were prepared from HEK GIRK1/2 cells using selection with Geneticin/G418 followed by single clone isolation. A stable monoclonal HEK GIRK1/4 expressing cell line was generated from HEK GIRK1 cells upon transfection with a pcDNA3.1 plasmid coding for GIRK4 using selection with Geneticin/G418. The cardiac muscle cell line HL-1 was purchased from Merck Millipore (Darmstadt, GER).

**Cell Culture.** All cell lines were cultivated with 5% CO2 at 37 °C in a humidified atmosphere. All media were supplemented with 10% (v/v) FCS, penicillin (100 U/mL), and streptomycin (100 μg/mL). Native HEK cells were cultivated in Dulbecco’s modified Eagle’s medium, and HEK cells stably transfected with GIRK subunits were cultivated in MEM Alpha Medium (1×) + GlutaMAX-I. For cell lines expressing the GIRK1 subunit, Blasticidin (final concentration of 5 μg/mL) was added to the media, Puromycin (3 μg/mL) for GIRK2, and Geneticin/G418 (400 μg/mL) for GIRK4 and OXE-R, respectively. HL-1 cells were cultivated in Claycomb medium supplemented with FCS 10% (v/v), penicillin (100 U/mL), norepinephrin (0.1 mM), and L-glutamine (2 mM).

**Label-Free Real-Time Biosensor Assays with Living Cells.** DMR was recorded with the Corning Epic biosensor as described previously.15 The Epic biosensor detects changes in extracellular impedance of a monolayer of cells located on the local index of refraction upon stimulus-induced mass redistribution within a cell monolayer that is grown in 384-well Epic microplates, which contain a resonant wave guide grating from the top of an electrical biosensor. The experiments were performed using an inverted brightfield phase-contrast microscope (DM IRE 2, Leica) equipped with a heated chamber (37 °C) and CO2 controller (5%) for 3 h after treatment. Pictures were taken every 3 min. Image processing was performed with ImageJ software.

**Thallium Flux Assay.** Activation of GIRK channels was measured using a Thallos AM dye-based thallium flux assay kit and recorded with the FlexStation 3 MultiMode Bench Top reader. The results show channel opening as increase in fluorescence over time. For the exact procedure, see the Supporting Information.

**Calcium Flux Assay.** Intracellular Ca2+ mobilization was measured using the calcium S assay kit (molecular devices) as previously described.58

**Cell Viability Assay.** Viability of HEK cell lines was assessed using the CellTiter-Blue assay from Promega following the manufacturer’s instructions. See the Supporting Information for more details.

**Data Analysis and Statistics.** All real-time recordings show one representative biological replicate as mean ± SEM of three technical replicates generated in parallel recordings. For quantification of DMR and CDS traces, at least three independent biological replicates were used, each consisting of three technical replicates. Concentration–effect curves were fitted using GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, CA, USA) showing means ± SEM and are based on the number of biological replicates indicated in the figure legends. P values were determined, and P < 0.05 was considered significant (**), P < 0.01 very significant (***), and P < 0.001 extremely significant (***)

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b02254.

Complete procedure for synthesis of Gore27, thallium flux assay, CellTiter-Blue assay, pEC50 values of ML297, pEC50 values for GIRK inhibitors, activity of ML297 analogs, GIRQ channel inhibitors, time-lapse imaging of native HEK, ML, analogs, and actin and microtubule inhibitors (PDF)

Time-lapse video files (ZIP)

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Notes
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