ASSAY and Drug Development Technologies
Volume 4, Number 5, 2006
© Mary Ann Liebert, Inc.

Technology Review

Cellular Dielectric Spectroscopy: A Label-Free Comprehensive Platform for Functional Evaluation of Endogenous Receptors

Edward Verdonk, Krystal Johnson, Ryan McGuinness, Gordon Leung, Yen-Wen Chen, H. Roger Tang, Julia M. Michelotti, and Vivian F. Liu

Downloaded by 70.181.107.185 from www.liebertpub.com at 10/13/25. For personal use only.

Abstract: The CellKey™* (MDS Sciex, South San Francisco, CA) system enables comprehensive pharmacological evaluation of cell surface receptors, including G-protein coupled receptors (GPCRs) and tyrosine kinase receptors, using adherent and suspension cell lines and primary cells. A unique application enabled by the ability of the CellKey system to reliably quantify activation of endogenous receptors is receptor panning. This application allows investigators to easily screen disease-relevant cell types for functionally active target receptors by treating cells with a panel of receptor-specific ligands. Receptor panning of multiple cell types including Chinese hamster ovary, human embryonic kidney 293, HeLa, U-937, U-2 OS, and TE671 cells resulted in the identification of many functionally active, differently coupled endogenous GPCRs, some of which have not been previously documented in the literature. Upon detecting GPCR activation in live cells, unique cellular dielectric spectroscopy (CDS) response profiles are generated within minutes that reflect the signaling pathways utilized and have been shown to be characteristic of Gs, Gq, and Gi GPCRs. The fact that the CDS response profiles are predictive of the G-protein coupling mechanism of the receptor was demonstrated by using examples of subtype-selective agonists/antagonists to identify the subtypes of the endogenous histamine and β-adrenergic receptors expressed in U-2 OS cells. A direct correlation is shown between receptor subtype G-protein coupling and CDS response profile. In addition, complex pharmacology, including detection of partial agonism and Schild analysis for endogenous receptors, is presented. The CellKey system allows investigators to conduct studies using endogenously expressed receptors to generate data that are physiologically relevant and in disease context.

Introduction

G-PROTEIN COUPLED RECEPTORS (GPCRs) represent the largest receptor target class in drug discovery, and they are important in therapeutic areas such as heart disease, metabolism, and immune disorders. Function is commonly assessed in cells artificially overexpressing the receptor of interest. Often, the overexpressed receptor is also coupled to a promiscuous G-protein or an artificially engineered reporter gene system using fluorescent- or bioluminescent-based probes. The ability to assess endogenous receptor function in their native setting may lead to information that is more biologically relevant than data collected from engineered cell lines. A key feature that distinguishes assays performed on the CellKey™ (MDS Sciex, South San Francisco, CA) system from other cell-based assays is the ability to reliably and robustly measure endogenous receptors that may be undetectable with other functional assays. The CellKey system has been used to perform comprehensive phar-

MDS Sciex, South San Francisco, CA.
*CellKey™ is a trademark of MDS Inc. through its MDS Sciex Division.

ABBREVIATIONS: cAMP, cyclic AMP; CDS, cellular dielectric spectroscopy; CGRP, calcitonin gene-related peptide; CHO, Chinese hamster ovary; CRF, corticotrophin-releasing factor; EC_{50}, 50% effective concentration; GPCR, G-protein coupled receptor; HEK, human embryonic kidney; 5-HT, 5-hydroxytryptamine (serotonin); IC_{50}, 50% inhibitory concentration; iec, extracellular current; itc, transcellular current; LPA, lysophosphatidic acid; TKR, tyrosine kinase receptor; VIP, vasoactive intestinal peptide.

Ex. 2027
Axion v. Agilent
IPR2024-01467

macological evaluation of cell surface receptors, including differently coupled GPCRs and tyrosine kinase receptors (TKRs) within adherent and suspension cell lines and primary cells.[1] In a single-assay format, cellular dielectric spectroscopy (CDS) monitors activation of different classes of endogenous GPCRs and simultaneously differentiates among those receptor classes. This is a large advantage over other cellular assays that require development of separate assays to monitor changes in secondary messengers, such as intracellular calcium (Ca^{2+}) or cyclic AMP (cAMP), or other universal assays that do not distinguish among the different GPCR subtypes.

CDS measures the changes in complex impedance (delta Z or dZ) of a cell layer that occur in response to receptor stimulation. Cells are seeded onto a CellKey standard 96-well microplate that contains electrodes at the bottom of the wells (Fig. 1). The CellKey instrument applies small voltages to these electrodes at 24 different measurement frequencies, once every 2 s. At low frequencies, these voltages induce extracellular currents (iec) that pass around individual cells in the layer. At high frequencies, they induce transcellular currents (itc) that penetrate the cellular membrane (Fig. 1). The ratio of the applied voltage to the measured current for each well is its impedance (Z) as described by Ohm's law.

When cells are exposed to a stimulus, such as a receptor ligand, signal transduction events are activated that lead to complex cellular events such as modulation of the actin cytoskeleton that cause changes in cell adherence, cell shape and volume, and cell-to-cell interaction. These cellular changes individually or collectively affect the flow of extracellular and transcellular current, and therefore, affect the magnitude and characteristics of the measured impedance.

Specifically, stimulation of differently coupled GPCRs generates differential effects on the actin cytoskeleton that affect the cellular parameters measured by impedance (Fig. 2). It has been shown that activation of Gq[2–4] and Gi GPCRs[5,6] and some TKRs[7,8] leads to increased actin polymerization, while stimulation of Gs GPCRs leads to actin depolymerization,[9–11] all within 30 s after receptor activation.[12,13] In addition, rapid changes in actin stress fiber formation are induced by direct modulators of second messengers involved in classical GPCR signaling pathways. For example, treating cells with agents that elevate the intracellular cAMP level causes marked alterations in cell morphology, including a rounded phenotype and a complete loss of actin microfilament bundles,[14–16] while treatment with agents that decrease the cAMP level results in increased actin stress fiber formation.[17] Phorbol ester-activated protein kinase C induced the early appearance of a great number of actin stress fibers.[18] CDS technology allows quantification of the changes in bioimpedance resulting from both cytoskeletal and non-cytoskeletal changes following receptor activation.

Materials and Methods

Cell lines

Chinse hamster ovary $(CHO)_{m1}$ (M1WT3; CRL-1985), HeLa (CCL-2), human embryonic kidney (HEK) 293 (CRL-1573), U-2 OS (HTB-96), TE671 (CCL-136), and U937 (CRL-1593.2)[19] cell lines were obtained from ATCC (Manassas, VA). CHO_{m1} cells were grown in Ham's F12K, HeLa and HEK293 cells were grown in Minimal Essential Medium, U-2 OS cells were grown in McCoy's 5a, TE671 cells were grown in Dulbecco's Modified Eagle's Medium, and U937 cells were grown in RPMI-1640. All media were supplemented with 10% fetal bovine serum, 50 μg/ml streptomycin, and 50 units/ml penicillin. CHO cells expressing transfected receptors (CHO_{m1}) were maintained under drug selection with 100 μg/ml G418.

Reagents

Reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. Somatostatin, adrenomedullin, calcitonin gene-related peptide (CGRP)-1, neurokinin B, pituitary adenylyl cyclase-activating polypeptide-38, and motilin were purchased from Calbiochem (San Diego, CA). (+)-Atenolol was purchased from Biomol (Plymouth Meeting, PA), and thioperamide and (2*R*,4*R*)-4-aminopyrrolidine-2,4-dicarboxylate were purchased from Tocris (Ellisville, MO). Human growth factor, keratinocyte growth factor, and β-nerve growth

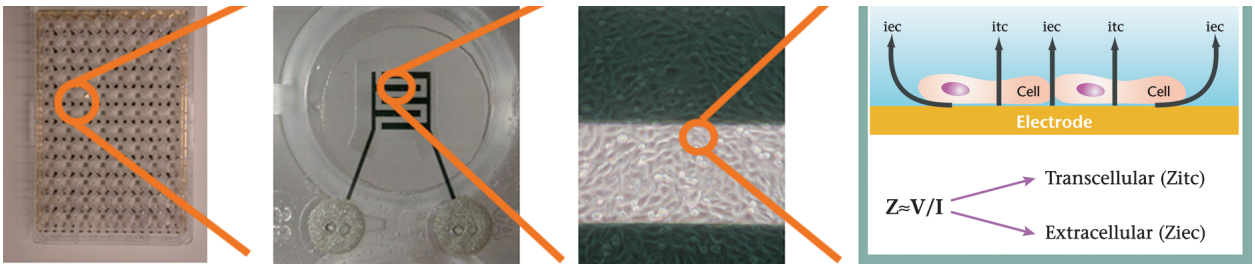


FIG. 1. CellKey standard 96-well microplate (**left**), assay well with a close-up of interdigitated gold electrodes (**center left**) with live cells plated on and between the electrode fingers (**center right**), and diagram highlighting flow of extracellular (iec) and transcellular (itc) current (**right**).

Downloaded by 70.181.107.185 from www.liebertpub.com at 10/13/25. For personal use only.

Ex. 2027
Axion v. Agilent
IPR2024-01467

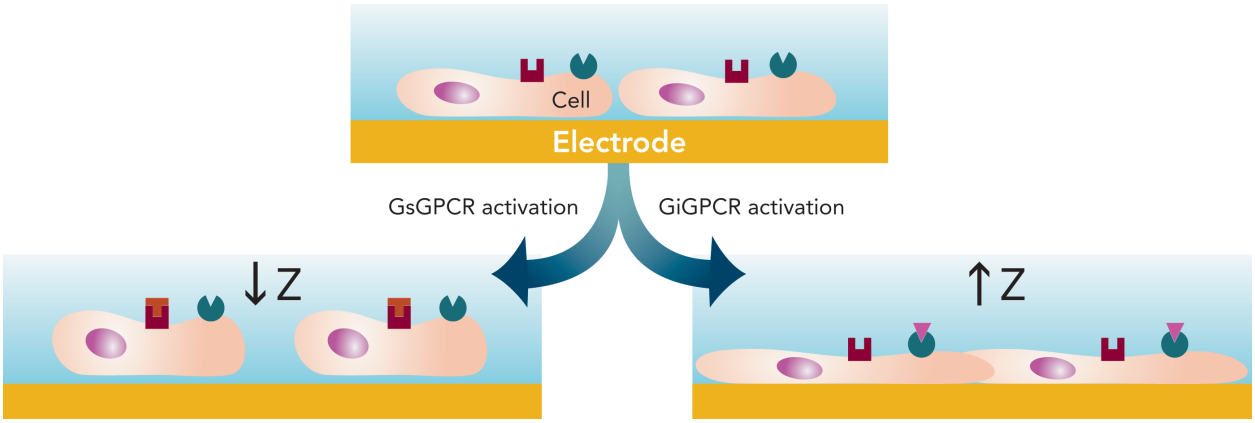


FIG. 2. Example of receptor-mediated changes in the cell that influence impedance measurements. Activation of Gi GPCRs leads to increased actin polymerization resulting in an increase in impedance, while stimulation of Gs GPCRs leads to actin depolymerization resulting in a decrease in impedance.

Downloaded by 70.181.107.185 from www.liebertpub.com at 10/13/25. For personal use only.

factor were purchased from R&D Systems (Minneapolis, MN).

Hanks' Balanced Salt Solution and HEPES Buffer Solution were purchased from Invitrogen (Grand Island, NY). Fetal bovine serum was purchased from Invitrogen (Grand Island, NY) and Gemini Bio-Products (Woodland, CA). Ham's F12K, Minimal Essential Medium, Dulbecco's Modified Eagle's Medium, McCoy's 5a, RPMI-1640, penicillin, streptomycin, and G418 were purchased from Cellgro (Herndon, VA).

The CellKey system

The CellKey system integrates a proprietary impedance measurement system, custom 96-well microtiter plates, onboard 96-well fluidics, and environmental control and custom acquisition and analysis software in a full solutions package (MDS Sciex) that is compatible with commercially available robotic platforms.

Small-amplitude AC voltage at 24 frequencies, from 1 KHz to 10 MHz, is applied to a monolayer of cells, and the resultant current is measured at an update rate of 2 s. The system is thermally regulated and experiments can be conducted between 28°C and 37°C. Onboard fluid additions and exchanges are handled by a 96-well head fluid delivery device with a range of 5–200 μl. In addition to the cell plate location, the CellKey system includes two additional stations for 96- or 384-well compound plates.

All aspects of assay set up, execution, and data acquisition and analysis are controlled by CellKey software.

Data in this study were collected from the CellKey system or prototypes of the CellKey system.

CellKey protocols

Adherent cells were seeded into the wells of CellKey standard 96-well microplates in standard tissue culture media containing serum, and the plates were incubated overnight under standard conditions (37°C, 5% CO_2). During the overnight incubation the cells adhered to, and formed a monolayer over, the electrodes at the bottom of the microplate, thus becoming part of an electric circuit. Typically, an average of 4×10^4 cells per well was used to form the monolayer, depending on the cell type. Growth medium is exchanged to buffer (Hanks' Balanced Salt Solution containing 20 m*M* HEPES and 0.1% bovine serum albumin) before the start of the experiment. Plates are placed onto the CellKey system, and pre-addition measurements are made for 5 min to obtain a baseline reading. Then, ligands are added simultaneously to all 96 wells using an integrated fluidics system. Activation of endogenous receptors results in changes in impedance that start occurring immediately after fluid addition and mixing. The quick update rate (2 s) of the CellKey system allows detection of these immediate changes in impedance. Impedance measurements are collected for 10 min after ligand addition to monitor cellular responses to ligand interaction and to generate CDS response profiles that are characteristic of the signaling mechanisms utilized. In the studies reported in this paper, experiments were conducted at 28°C.

For experiments with non-adherent cells, cells were harvested from stock tissue culture flasks and washed by centrifugation three times with buffer. Typically an average of 1×10^5 cells were then added to each well and allowed to settle for 30 min at room temperature. This resulted in a single layer of cells on top of the electrodes. After the cells settled, the cell plate was placed on the instrument, and experiments were initiated in the manner described above for adherent cell assays. For non-adherent cells, the pipetting speed and pipette tip height of the fluidics head were adjusted so as not to disturb the monolayer of settled cells. Basic protocols for running adherent and non-adherent cell assays that can be further refined by the user are included in the CellKey software.

Ex. 2027
Axion v. Agilent
IPR2024-01467

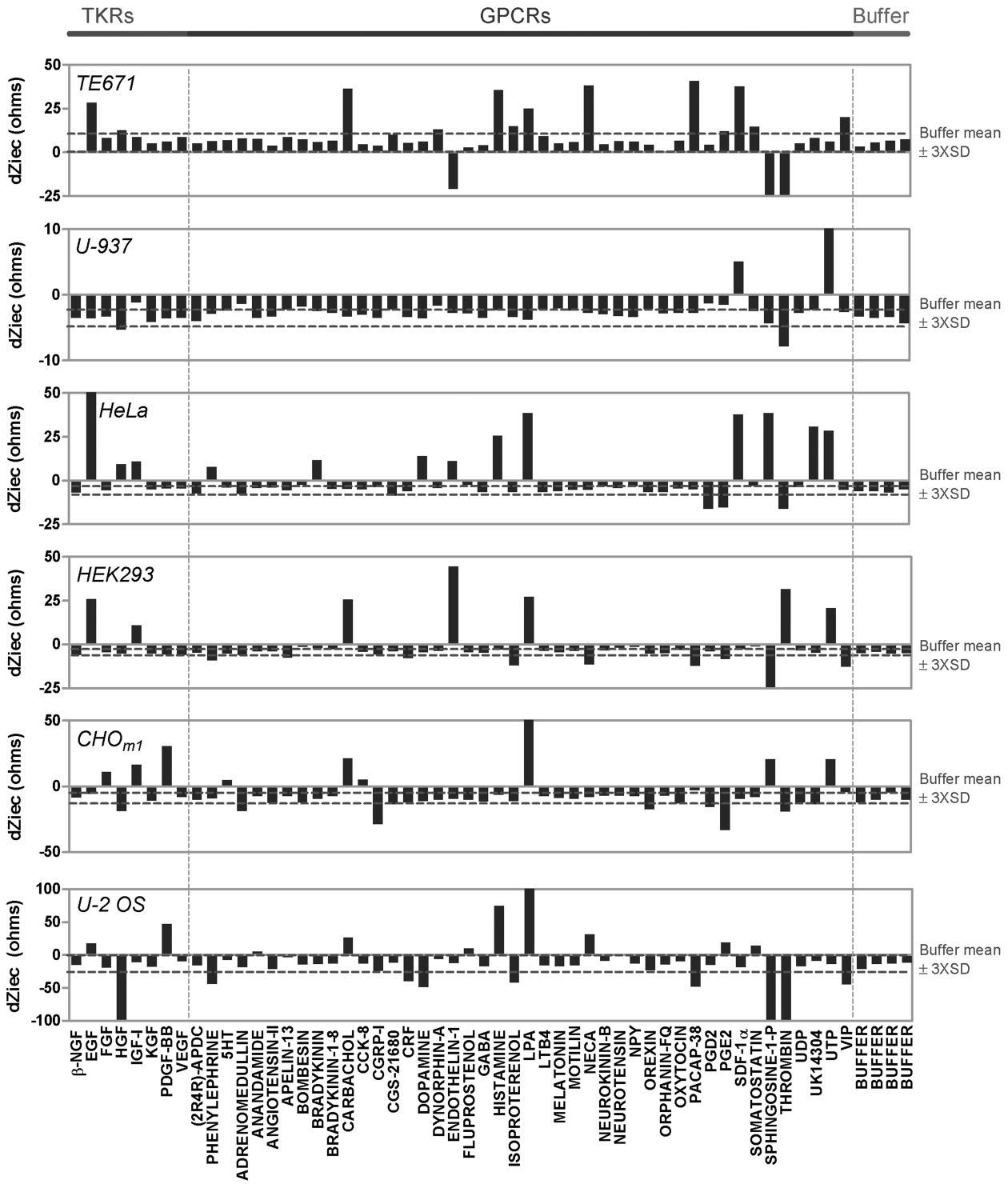


FIG. 3. CDS responses identify functional endogenous receptors in cells treated with a panel of ligands targeting GPCRs and TKRs at 28°C. The ligand-induced response is measured as the maximum deviation from a baseline measurement. Those receptor ligands that yield CDS responses in terms of dZiec that are 3 standard deviations (SD) above and below the buffer mean (dotted lines) are considered statistically significant and indicative of functionally active endogenous receptors. β-NGF, β-nerve growth factor; EGF, epidermal growth factor; FGF, fibroblast growth factor; HGF, human growth factor; IGF-1, insulin-like growth factor-1; KGF, keratinocyte growth factor; PDGF-BB, platelet-derived growth factor BB; VEGF, vascular endothelial growth factor; (2*R*4*R*)-APDC, (2R,4R)-4-aminopyrrolidine-2,4-dicarboxylate; CCK-8, cholecystokinin octapeptide; GABA, γ-aminobutyric acid; LTB4, leukotriene B4; NECA, 5′-*N*-ethylcarboxamidoadenosine; NPY, neuropeptide Y; PACAP-38, pituitary adenylate cyclase activating polypeptide 38; PGD2, prostaglandin D2; PGE2, prostaglandin E2; SDF-1α, stromal cell-derived factor-1α.

Downloaded by 70.181.107.185 from www.liebertpub.com at 10/13/25. For personal use only.

Ex. 2027
Axion v. Agilent
IPR2024-01467

Results

Cataloguing active endogenous receptors in different cell types

The unique ability of the CellKey system to measure functional activation of endogenous receptors enables screening and pharmacological evaluation of compounds targeting cell surface receptors in a native setting. Receptor panning is a novel application enabled by the CellKey system that allows rapid assessment of the endogenous receptors that are functionally active in different cells to facilitate selection of relevant cells or cell lines in which to assess the receptor(s) of interest.

Receptor panning involves treating cells with a panel of generic and receptor-specific ligands, compounds, and peptides known to activate different types of receptors of interest. Changes in impedance related to extracellular current (dZiec) are measured before and after exposure to ligands. The ligand-induced response is measured as the maximum deviation from a baseline measurement, and this response can be either positive or negative because the CellKey platform measures both increases and decreases in impedance that occur following receptor activation. Those receptor ligands that yield CDS responses in terms of dZiec that are 3 standard deviations above and below the buffer mean (negative control) are considered statistically significant and indicative of functionally active endogenous receptors. Using a series of bioactive ligands, functional activation of a wide range of TKRs and differently coupled GPCRs can be detected on a single plate in a single-assay format for each cell line of interest just by measuring the change in impedance that occurs upon ligand addition.

To illustrate the use of the receptor panning application, adherent cells (CHO, HEK293, HeLa, TE671, and U-2 OS) and suspension cells (U-937) that are commonly used in drug discovery were treated with a panel of ligands targeting GPCRs and TKRs to catalog the active endogenous receptors in each cell line. Ligand-induced activation of a wide range of GPCRs and TKRs were differentially detected in the cell lines tested (Fig. 3). Some ligands such as thrombin generate a positive response in most or all cells tested, whereas other ligands generate a positive response in only one cell line. For example, 5-hydroxytryptamine (5-HT; serotonin) activates the endogenous 5-HT_{1B} receptor in CHO_{m1} cells but did not elicit a response in the other cells tested in this study. In addition, CHO_{m1} cells appear to be the only cell line tested here that expresses the fibroblast growth factor receptor. The addition of epidermal growth factor elicits a positive response in HeLa, HEK293, U-2 OS, and TE671 cells but not in CHO_{m1} or U-937 cells. A list of the functionally active endogenous receptors identified by receptor panning of the cells tested is available in Table 1. The data shown here indicate that the CellKey system can quickly and easily detect a variety of differentially active endogenous receptors and receptor classes in the context of a variety of cell types.

The magnitude of the CDS response is dependent primarily upon how tightly the receptor is coupled to the signaling mechanism upon ligand stimulation, and less dependent upon receptor expression levels. For example, the magnitude of the transfected muscarinic m_1 receptor response in CHO_{m1} cells is not significantly different from those pertaining to the endogenous receptors (serotonin 5-HT_{1B}, CGRP, P2Y, and prostanoid EP_4) in the same cells. The CHO_{m1} cells (M1WT3) are reported to express 800 fmol of receptor protein/mg of membrane protein,[20] while expression of the 5-HT_{1B} receptor in CHO cells is too low to be detected by radioactive ligand binding assays.[21] However, functional assays such as intracellular cAMP and CDS can be used to detect activation of this low-density receptor that is very efficiently coupled to its effector.[21,22] The sensitivity of CDS to receptor-mediated cytoskeletal and an integrated cellular response enables robust and reliable measurement of many endogenous cell surface receptors.

Table 1. Partial List of Active Endogenous Receptors Identified by Receptor Panning

Cell	*GPCRs*[a]	*TKRs*[b]
TE671	Muscarinic m_3,[23] ET, histamine, β-adrenergic, LPA, sphingolipid, thrombin, adenosine, opioid, VIP, somatostatin, prostanoid, CXCR-4	EGF, HGF
U-937	P2Y, CXCR-4	
HeLa	α1- and α2-adrenoceptors, bradykinin, dopamine, ET, histamine, LPA, sphingolipid, thrombin, prostanoid, P2Y, CXCR-4	EGF, HGF, IGF-1
HEK293	α1- and β2-adrenoceptors,[24] muscarinic m_3, ET,[24] LPA, VIP, sphingolipid, thrombin, P2Y,[24] adenosine A_{2B}[24]	EGF, IGF-1
CHO_{ml}	5-HT_{1B},[21] (muscarinic m_1, transfected), prostanoid, LPA, sphingolipid, thrombin, P2Y, CGRP	FGF, HGF, IGF-1, PDGF
U-2 OS	Histamine, LPA, sphingolipid, thrombin, prostanoid, dopamine, β2-adrenoceptor, muscarinic, adenosine, VIP, CRF, somatostatin	HGF, EGF, PDGF

[a]ET, endothelin.

[b]EGF, epidermal growth factor; HGF, human growth factor; IGF-1, insulin-like growth factor-1; PDGF, platelet-derived growth factor.

Downloaded by 70.181.107.185 from www.liebertpub.com at 10/13/25. For personal use only.

Ex. 2027
Axion v. Agilent
IPR2024-01467

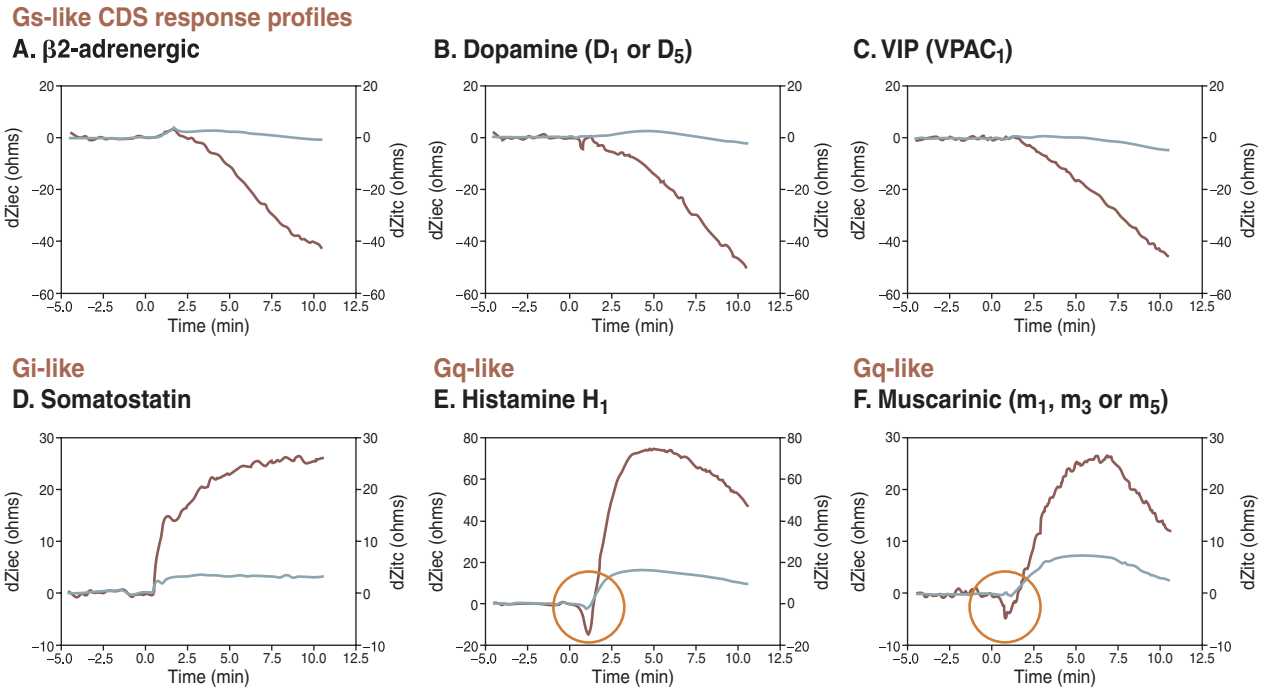


FIG. 4. CDS response profiles of differently coupled GPCRs in U-2 OS cells. (**A–C**) Activation of the β-adrenergic (A), dopamine (B), and VIP (C) receptors were stimulated by addition of isoproterenol (10 μM), dopamine (10 μM), and VIP (633 nM), respectively, and yielded CDS response profiles that were typical of Gs GPCRs. (**D**) The endogenous somatostatin receptor was stimulated by addition of somatostatin (10 μM) to generate a response profile typical of Gi GPCRs. (**E** and **F**) Cells were treated with histamine (10 μM; E) and carbachol (10 μM; F) to stimulate the endogenous histamine H_1 and muscarinic receptors, respectively, that both yielded Gq-like CDS response profiles. The early transient dip in impedance followed by the later increase in impedance that is characteristic of Gq GPCRs is circled. dZiec, changes in current due to extracellular current. dZitc, changes in current due to transcellular current.

Identifying the G-protein coupling mechanism of GPCRs

Few reports in the literature document the expression of endogenous receptors in U-2 OS cells. However, receptor panning showed that the following GPCRs were functionally active in U-2 OS cells: muscarinic, prostanoid, thrombin, adenosine, corticotropin-releasing factor (CRF), dopamine, β-adrenergic, histamine, lysophosphatidic acid (LPA), sphingolipid, vasoactive intestinal peptide (VIP), and somatostatin receptors.

To increase understanding of these endogenous receptors expressed in U-2 OS cells, we next focused on identifying their respective G-protein coupling mechanisms. We have previously established that upon detecting GPCR activation, the CellKey system generates unique CDS response profiles that reflect the signaling pathways utilized in the cells and are characteristic of Gs, Gq, and Gi GPCRs.[1] The CDS response profiles of GPCRs are similar in many cell types, including CHO, HeLa, HEK293, and U-2 OS cells. These data suggest that differently coupled GPCRs generate distinct CDS response profiles that are characteristic of the signaling mechanisms used during receptor activation. Comparing the CDS response profiles of known GPCRs can be used to provide an indication of the signaling mechanism of unknown GPCRs.

A few examples of CDS response profiles generated upon ligand addition to activate endogenous receptors in U-2 OS cells are shown in Fig. 4. Addition of isoproterenol, dopamine, or VIP resulted in CDS response profiles typical of Gs GCPRs, consisting of a decrease in impedance, suggesting that the endogenous β-adrenergic, dopamine, and VIP receptors in U-2 OS cells are Gs-coupled (Fig. 4A–C). Addition of somatostatin to activate the endogenous somatostatin receptor in U-2 OS generated a CDS response profile typical of Gi GPCRs consisting of an immediate increase in impedance (Fig. 4D). The Gi-like nature of somatostatin receptor response profile is consistent with the fact that all known subtypes of the somatostatin receptor are Gi-coupled.[25] What distinguishes the Gq GPCRs from the Gi GPCRs is an initial transient dip in impedance that is followed by a later increase in impedance.[1] Activation of the endogenous histamine and muscarinic receptors with addition of histamine or carbachol, respectively, generated CDS response profiles that are characteristic of Gq GPCRs (Fig. 4E and F), suggesting that these receptors are Gq-coupled in

Downloaded by 70.181.107.185 from www.liebertpub.com at 10/13/25. For personal use only.

Ex. 2027
Axion v. Agilent
IPR2024-01467

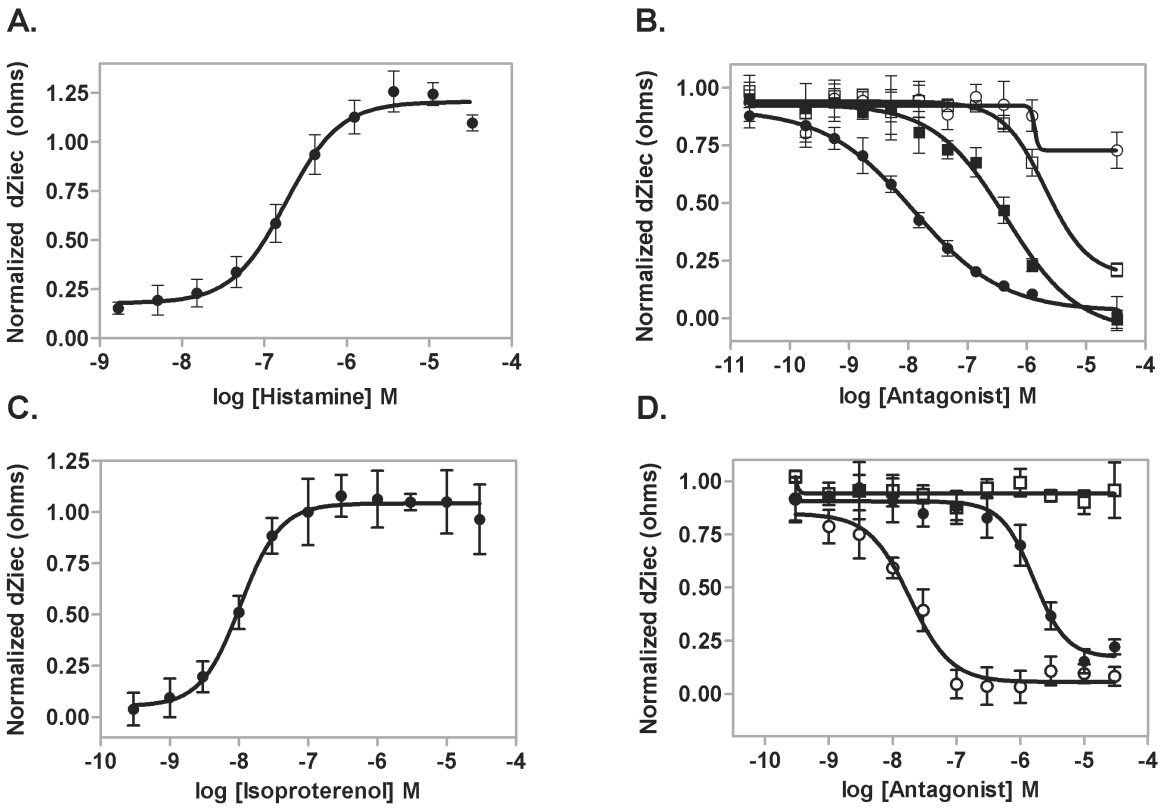


FIG. 5. Characterizing the endogenous histamine and β-adrenergic receptor subtypes in U-2 OS cells. (**A**) Concentration–response curve of histamine targeting the endogenous histamine receptor yields an EC_{50} value of 182 n*M* in U-2 OS cells. (**B**) Concentration–response curves of antagonists [triprolidine (●); fluphenazine (■); cimetidine (❒); and thioperamide (○)] targeting different histamine receptor subtypes in U-2 OS cells challenged with 10 μM histamine. Responses were normalized to 10 μM histamine. The IC_{50} values of the histamine H_1 receptor antagonists triprolidine and fluphenazine are 9.7 n*M* and 440 n*M*, respectively. The H_2 subtype-selective antagonist (cimetidine) and an H_3-, H_4-subtype-selective antagonist (thioperamide) were unable to achieve blocking at physiological concentrations. (**C**) Concentration–response curve of isoproterenol targeting the endogenous β-adrenergic receptor yields an EC_{50} value of 10.6 n*M* in U-2 OS cells. (**D**) Concentration–response curves of antagonists targeting different β-adrenergic receptor subtypes in U-2 OS cells challenged with 100 n*M* isoproterenol. Responses were normalized to 100 n*M* isoproterenol. The subtype-selective β2- and (β3-antagonists ICI-118,551 (○) and SR 59230A (●) had IC_{50} values of 18.7 n*M* and 1.6 μM, respectively, while the β1-antagonist atenolol (❒) did not block the response induced by isoproterenol. Data represent mean ± standard deviation values ($n = 3$).

Downloaded by 70.181.107.185 from www.liebertpub.com at 10/13/25. For personal use only.

U-2 OS cells. Thus, not only does the CellKey system enable analyses of receptors in their natural settings, but this platform also generates CDS response profiles that provide an indication of the receptor signaling mechanisms.

Receptor subtyping

In this study, generic receptor ligands that targeted the majority of the subtypes for each receptor family were used for receptor panning to maximize the chances of identifying active endogenous receptors in each cell line (Fig. 3). Addition of histamine to U-2 OS cells during receptor panning resulted in a positive response significantly above buffer background, suggesting that the histamine receptor is functionally active in these cells (Fig. 3). Treating U-2 OS cells with varying concentrations of histamine resulted in a 50% effective concentration (EC_{50}) value of 182 n*M* (Fig. 5A), which is within the range of published values for the endogenous H_1 receptor in different systems such as isolated guinea-pig heart (54 n*M*),[26] cultured bovine corneal endothelial cells (0.84 μM),[27] and HeLa cells (8.6 μM).[28] EC_{50} and 50% inhibitory concentration (IC_{50}) values determined using the CellKey system are reproducible across separate experiments, resulting in values that fall within a half-log of each other.

The process of identifying the specific receptor subtype expressed in a cell is simplified by the ability to analyze real-time kinetic CDS response profiles. For example, the shape of the CDS response profile, characterized by an initial transient dip followed by a later increase in impedance that was generated upon addition of hista-

Ex. 2027
Axion v. Agilent
IPR2024-01467

mine to U-2 OS cells (Fig. 4E), suggested that these cells express a histamine receptor subtype that is Gq-coupled. Of the four known subtypes of the histamine receptor, only H_1 is Gq-coupled, whereas H_2 is Gs-coupled, H_3 is Gi-coupled, and H_4 is Gi- and G15/16-coupled. The Gq-like format of the CDS response profile suggests that U-2 OS cells express the H_1 subtype. This was confirmed by demonstrating that two H_1 subtype-selective antagonists were able to block the cells' response to 10 μM histamine, whereas an H_2 subtype-selective antagonist and an H_3 and H_4 subtype-selective antagonist were unable to achieve blocking at physiologic concentrations (Fig. 5B).

Addition of isoproterenol to U-2 OS cells generated a positive response in the receptor panning experiments, suggesting that an endogenous β-adrenergic receptor is functionally active in these cells. Treating U-2 OS cells with a range of isoproterenol concentrations yielded an EC_{50} value of 10.6 nM, which is in agreement with published values for the endogenous β-adrenoceptor in other endogenous systems.[29,30] Activation of the β-adrenergic receptor elicited a CDS response with a Gs-like profile characterized by a decrease in impedance (Fig. 4A). Because all three known subtypes of β-adrenoceptors are Gs-coupled, the response profile did not provide an indication of the subtype expressed in these cells. Subtype-selective antagonists were then used to determine that the β2-subtype is functionally active in U-2 OS cells. As shown in Fig. 5D, the β2-selective antagonist ICI 118,551 blocked the isoproterenol-induced response with an IC_{50} value of 18.7 nM. Meanwhile, concentrations in the micromolar range were required for the β3-selective antagonist SR 59230A to block the β-adrenoceptor response, and atenolol, a β1-selective antagonist, was unable to block the response elicited by isoproterenol. These data suggest that the β2-adrenoceptor is functionally active in U-2 OS cells. The CellKey system is sensitive enough to provide information regarding the presence of functionally active endogenous receptors such as the β2-adrenoceptor in U-2 OS cells that have not been previously documented in the literature. In fact, studies had been published focusing on the transfected version of the β2-adrenoceptor in these U-2 OS cells that are shown here to contain a functionally active endogenous form of the same receptor.[31,32]

The CDS response profiles also provide indications of the G-protein coupling mechanisms utilized upon addition of VIP, dopamine, or carbachol to U-2 OS cells. The three currently characterized VIP receptors signal via Gs- ($VPAC_1$), Gs/Gq- ($VPAC_2$), and Gq- (PAC_1) coupled mechanisms. The Gs-like response profile that is generated upon addition of VIP to U-2 OS cells suggests that the subtype expressed in these cells is the $VPAC_1$ subtype. In a similar manner, the dopamine receptor subtype expressed in these cells can be deduced to be one of the two Gs-coupled subtypes (D_1 or D_5) because the remaining subtypes (D_2, D_3, and D_4) are Gi-coupled. The CDS response profile indicates that the muscarinic receptor subtype expressed in U-2 OS cells is Gq-coupled, and not Gi-coupled (Fig. 4F). The muscarinic receptor response was insensitive to pretreatment of the cells with a Gi inhibitor, pertussis toxin (data not shown), which confirmed that the subtype expressed in U-2 OS cells is not Gi-coupled. Thus, the muscarinic receptor subtype in U-2 OS cells is likely to be one of the Gq-coupled subtypes, which include the m_1, m_3, and m_5 subtypes. The use of subtype-selective agonists and/or antagonists can then be used to confirm or refine the determination of the subtypes expressed in U-2 OS cells.

Pharmacology

The data presented thus far demonstrate that CDS is a powerful tool for screening of mammalian cell lines for endogenous receptors with minimal assay development and without fluorescent or bioluminescent-based probes. In addition to detecting and characterizing activation of endogenous receptors, the CellKey system can also be used to generate relevant pharmacological data, such as potency ranking (Fig. 5) for ligands targeting these endogenous receptors. CDS generates pharmacological data that are comparable to data generated from assays designed to measure changes in intracellular second messengers. For example, CDS generates the same potency rankings as calcium mobilization for agonists targeting the endogenous P2Y receptor in U-937 cells.[1] CDS has also been used to generate EC_{50} values measuring the functional effect of interleukin-8 on the endogenous CXCR1 and CXCR2 receptors in primary human neutrophils that were in agreement with values obtained from

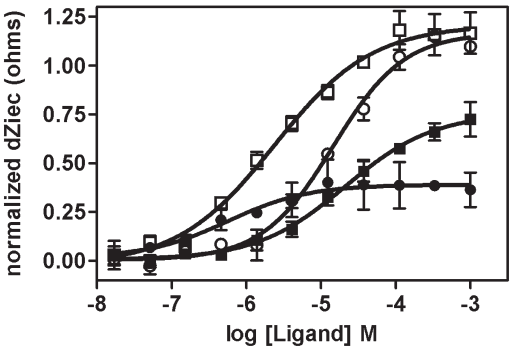


FIG. 6. Concentration–response curves of agonists targeting the endogenous muscarinic receptor in U-2 OS cells. Cells were treated with the following muscarinic agonists: carbachol (○), oxotremorine M (❐), arecaidine propargyl ester (●), and arecoline (■). CDS responses were normalized to 100 μM carbachol. Data represent mean ± standard deviation values ($n = 3$).

Downloaded by 70.181.107.185 from www.liebertpub.com at 10/13/25. For personal use only.

Ex. 2027
Axion v. Agilent
IPR2024-01467

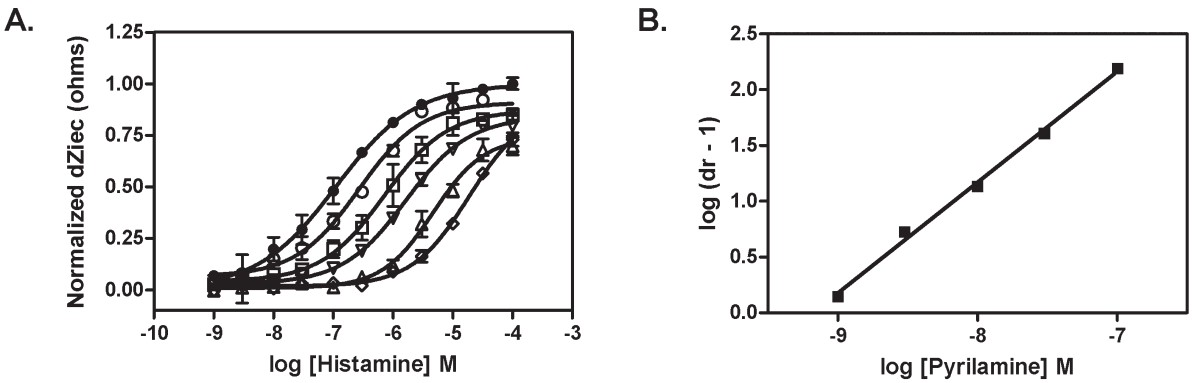


FIG. 7. Schild analysis with histamine agonist and pyrilamine antagonist on U-2 OS cells. (**A**) Cells were pretreated with different concentrations of the antagonist pyrilamine [no pyrilamine (●); 1 n*M* (○); 3 n*M* (❒); 10 n*M* (▽); 30 n*M* (△); or 100 n*M* (◇) prior to stimulation with varying concentrations of histamine. As the concentration of antagonist was increased, a rightward shift in the EC_{50} of the agonist was observed. CDS responses were normalized to 10 μM histamine. Data represent mean ± standard deviation values ($n = 3$). (**B**) From the relationship between the shifted EC_{50} values and the concentration of antagonist, the pA_2 value was determined to be 9.1 ($r^2 = 0.996$).

Downloaded by 70.181.107.185 from www.liebertpub.com at 10/13/25. For personal use only.

chemotaxis assays. The ability to measure activation of the CXCR receptors in the neutrophils provided a demonstration of the sensitivity of the CDS technology to assaying an endogenous receptor system that is not amenable to traditional Ca^{2+} flux or cAMP assays.[1]

Partial agonism. The CellKey system's ability to measure a range of increases and decreases in impedance as a result of GPCR activity suggests that in addition to measuring potency, the efficacy of ligands targeting endogenous receptors can also be assessed. To provide a demonstration of how the platform can be used to distinguish partial agonism from full agonism for ligands targeting an endogenous receptor, U-2 OS cells expressing the endogenous muscarinic receptor were treated with ligands previously characterized as full or partial agonists to generate the concentration–response curves shown in Fig. 6. Arecaidine and arecoline exhibited partial agonism, while oxotremorine-M and carbachol displayed full agonism in the U-2 OS cells. These data are consistent with previously published information about the full and partial agonist effects of these ligands targeting both the endogenous m_3[33] and transfected muscarinic m_1 receptors.[34]

Schild analysis. We have previously shown that CDS can be used to generate pA_2 values for the transfected dopamine D_1 receptor in CHO cells.[1] Here we provide a demonstration that Schild analysis can also be extended to antagonists targeting an endogenous receptor using the CellKey system. U-2 OS cells expressing a functionally active endogenous histamine H_1 receptor were pretreated with various concentrations of the classical H_1 antagonist, pyrilamine, prior to the generation of histamine concentration–response curves (Fig. 7A). From the relationship between the shifted EC_{50} values and the concentration of antagonist, the pA_2 value of pyrilamine was determined to be 9.1 (Fig. 7B), which is in close agreement with the pA_2 value of 8.9 based on [^{3}H]inositol trisphosphate accumulation.[35]

Thus, in addition to ranking compounds in terms of potency and efficacy, the CellKey system can also be used to generate pA_2 values for antagonists targeting endogenous receptors via Schild analysis. These data demonstrate both the sensitivity and ease of use of the CellKey system in generating relevant pharmacological data for endogenous receptors in a universal and simple assay format.

Discussion

The frequency of cell-based assays usage in the context of drug discovery for GPCR targets is increasing as such assays are utilized in primary and secondary screening and lead evaluation and optimization. The challenging aspects of traditional cell-based GPCR assays including the requirement for overexpressing the GPCR target and development of different types of assays for differently coupled GPCRs are circumvented by the CellKey system because of its ability to sensitively measure cellular changes that occur upon GPCR activation regardless of G-protein coupling mechanism. The rich kinetic and physiologically relevant nature of the CellKey data underlies a wide range of applications including receptor panning, analysis of signaling pathways, and complex pharmacology. These applications and the 96-well format are more amenable to target

Ex. 2027
Axion v. Agilent
IPR2024-01467

identification and validation, hit confirmation or secondary screening, and lead validation and optimization than primary or high throughput screening. However, the ability of the CellKey system to reliably and robustly measure endogenous receptors is truly enabling, allowing users to screen smaller focused libraries on the order of tens of thousands of compounds for ligands targeting an endogenous receptor that may be patent-protected or otherwise unavailable for transfection and overexpression.

The CellKey system enables the screening of mammalian cell lines for endogenous receptors with minimal assay development and without fluorescent or bioluminescent-based probes. Receptor panning of cell lines frequently used in drug discovery has resulted in the identification of functionally active and differently coupled endogenous GPCRs, many of which have not been previously documented in the literature for these cells. Of particular interest is the fact that studies are frequently conducted with receptors transfected into CHO, HEK293, and U-2 OS cells that actually express many of these same receptors or related subtypes endogenously. For example, receptor panning and subsequent analysis with subtype-selective agonists indicate that an endogenous β2-adrenergic receptor is activated by addition of isoproterenol in U-2 OS cells, yet studies have published using arrestin translocation to generate EC_{50} values for agonists on the transfected β2-adrenoceptor in U-2 OS cells.[31,32] Even though CHO cells express a functionally active 5-HT_{1B} receptor[21] (Fig. 3), studies have been performed using transfected versions of the following 5-HT receptor subtypes in CHO cells: $5HT_{1A}$,[36–39] 5-HT_{1D},[40] 5-HT_7,[41] and 5-HT_4.[42] A series of antiparkinsonian agents were evaluated for their interactions within CHO cells transfected with the 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{2A}, or 5-HT_{2C} receptors.[43] Studying the effects of ligands on transfected receptors in cells already expressing endogenous versions of either the same receptor or related subtypes may lead to confounding results upon ligand binding and activation of the multiple subtypes.

The ability to monitor endogenous receptor activation provides several advantages, including the generation of biologically relevant data, freedom from lengthy and expensive DNA and cell cloning procedures, and the ability to investigate systems whose recombinant genes are patent-protected. In addition, determining the profile of active receptors in a cell can be used to characterize the state of a cell based on disease, origin, tissue, state of differentiation, and exposure to compounds. This is in contrast to methods such as gene expression and protein profiling, which only yield information about relative amounts of messenger RNA and protein in different cell contexts and require cumbersome amplification and detection techniques. Instead, disease relevant cell types can be screened for functionally active target receptors or biomarkers of interest. The ability to quickly and easily determine the profile of active receptors in any given cell may be of great use in a variety of fields ranging from research into disease processes to diagnostics.

References

1. Leung G, Tang HR, McGuinness R, Verdonk E, Michelotti JM, Liu VF: Cellular dielectric spectroscopy: a label-free technology for drug discovery. *J Assoc Lab Automation* 2005;10:258–269.
2. Kawanabe Y, Okamoto Y, Nozaki K, Hashimoto N, Miwa S, Masaki T: Molecular mechanism for endothelin-1-induced stress-fiber formation: analysis of G proteins using a mutant endothelinA receptor. *Mol Pharmacol* 2002;61: 277–284.
3. Lesh RE, Emala CW, Lee HT, Zhu D, Panettieri RA, Hirshman CA: Inhibition of geranylgeranylation blocks agonist-induced actin reorganization in human airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2001; 281:L824–L831.
4. Pierce KL, Fujino H, Srinivasan D, Regan JW: Activation of FP prostanoid receptor isoforms leads to Rho-mediated changes in cell morphology and in the cell cytoskeleton. *J Biol Chem* 1999;274:35944–35949.
5. Schraufstatter IU, Chung J, Burger M: IL-8 activates endothelial cell CXCR1 and CXCR2 through Rho and Rac signaling pathways. *Am J Physiol Lung Cell Mol Physiol* 2001;280:L1094–L1103.
6. Saltarelli D: Heterotrimetric Gi/o proteins control cyclic AMP oscillations and cytoskeletal structure assembly in primary human granulosa-lutein cells. *Cell Signal* 1999;11: 415–433.
7. Lorenz M, DesMarais V, Macaluso F, Singer R, Condeelis J: Measurement of barbed ends, actin polymerization, and motility in live carcinoma cells after growth factor stimulation. *Cell Motil Cytoskeleton* 2004;57:207–217.
8. Maddala R, Reddy V, Epstein D, Rao V: Growth factor induced activation of Rho and Rac GTPases and actin cytoskeletal reorganization in human lens epithelial cells. *Mol Vis* 2003;9:329–336.
9. Martineau LC, McVeigh LI, Jasmin BJ, Kennedy CRJ: p38 MAP kinase mediates mechanically induced COX-2 and PG EP_4 receptor expression in podocytes: implications for the actin cytoskeleton. *Am J Physiol Renal Physiol* 2004; 286:F693–F701.
10. Perez V, Bouschet T, Fernandez C, Bockaert J, Journot L: Dynamic reorganization of the astrocyte actin cytoskeleton elicited by cAMP and PACAP: a role for phosphatidylinositol 3-kinase inhibition. *Eur J Neurosci* 2005;21:26–32.
11. Suzuki H, Nakamura I, Takahashi N, Ikuhara T, Matsuzaki K, Isogai Y, et al.: Calcitonin-induced changes in the cytoskeleton are mediated by a signal pathway associated with protein kinase A in osteoclasts. *Endocrinology* 1996;137:4685–4690.
12. Li Z, Kim ES, Bearer EL: Arp2/3 complex is required for actin polymerization during platelet shape change. *Blood* 2002;99:4466–4474.
13. Dittmar T, Husemann A, Schewe Y, Nofer J-R, Niggemann B, Zanker KS, *et al*.: Induction of cancer cell migration by epidermal growth factor is initiated by specific phosphorylation of tyrosine 1248 of c-erbB-2 receptor via epidermal growth factor receptor. *FASEB J* 2002;16:1823–1825.
14. Lamb N, Fernandez A, Conti M, Adelstein R, Glass D, Welch W, *et al*.: Regulation of actin microfilament integrity in living nonmuscle cells by the cAMP-dependent protein

Downloaded by 70.181.107.185 from www.liebertpub.com at 10/13/25. For personal use only.

Ex. 2027
Axion v. Agilent
IPR2024-01467

Downloaded by 70.181.107.185 from www.liebertpub.com at 10/13/25. For personal use only.

kinase and the myosin light chain kinase. *J Cell Biol* 1988; 106:1955–1971.
15. Kreisberg JI, Ghosh-Choudhury N, Radnik RA, Schwartz MA: Role of Rho and myosin phosphorylation in actin stress fiber assembly in mesangial cells. *Am J Physiol Renal Physiol* 1997;273:F283–F288.
16. Szaszi K, Kurashima K, Kaibuchi K, Grinstein S, Orlowski J: Role of the cytoskeleton in mediating cAMP-dependent protein kinase inhibition of the epithelial Na^+/H^+ exchanger NHE3. *J Biol Chem* 2001;276:40761–40768.
17. Patterson C, Lum H, Schaphorst K, Verin A, Garcia J: Regulation of endothelial barrier function by the cAMP-dependent protein kinase. *Endothelium* 2000;7:287–308.
18. Gujdar A, Sipeki S, Bander E, Buday L, Farago A: Phorbol ester-induced migration of HepG2 cells is accompanied by intensive stress fibre formation, enhanced integrin expression and transient down-regulation of p21-activated kinase 1. *Cell Signal* 2003;15:307–318.
19. Sundstrom C, Nilsson K: Establishment and characterization of a human histiocytic lymphoma cell line (U-937). *Int J Cancer* 1976;17:565–77.
20. Buck M, Fraser C: Muscarinic acetylcholine receptor subtypes which selectively couple to phospholipase C: pharmacological and biochemical properties. *Biochem Biophys Res Commun* 1990;173:666–672.
21. Giles H, Lansdell S, Bolofo M, Wilson H, Martin G: Characterization of a 5-HT1B receptor on CHO cells: functional responses in the absence of radioligand binding. *Br J Pharmacol* 1996;117:1119–1126.
22. Ciambrone GJ, Liu VF, Lin DC, McGuinness RP, Leung GK, Pitchford S: Cellular dielectric spectroscopy: a powerful new approach to label-free cellular analysis. *J Biomol Screen* 2004;9:467–480.
23. Bencherif M, Lukas R: Ligand binding and functional characterization of muscarinic acetylcholine receptors on the TE671/RD human cell line. *J Pharmacol Exp Ther* 1991; 257:946–953.
24. Mundell SJ, Benovic JL: Selective regulation of endogenous G protein-coupled receptors by arrestins in HEK293 cells. *J Biol Chem* 2000;275:12900–12908.
25. Watling KJ: *The Sigma-RBI Handbook of Receptor Classification and Signal Transduction*, Sigma-Aldrich Research Biochemicals Inc., Natick, MA, 2001.
26. Trzeciakowski J, Levi R: Reduction of ventricular fibrillation threshold by histamine: resolution into separate H1- and H2-mediated components. *J Pharmacol Exp Ther* 1982;223:774–783.
27. Crawford K, MacCallum D, Ernst S: Histamine H_1 receptor-mediated Ca^{2+} signaling in cultured bovine corneal endothelial cells. *Invest Ophthalmol Vis Sci* 1992;33: 3041–3049.
28. Raymond J, Albers F, Middleton J, Lefkowitz R, Caron M, Obeid L, et al.: 5-HT1A and histamine H1 receptors in HeLa cells stimulate phosphoinositide hydrolysis and phosphate uptake via distinct G protein pools. *J Biol Chem* 1991;266:372–379.
29. Gross S, Mayer S, Longshore M: Stimulation of glycogenolysis by beta adrenergic agonists in skeletal muscle of mice with the phosphorylase kinase deficiency mutation (I strain). *J Pharmacol Exp Ther* 1976;198:526–538.
30. Rump LC, Bohmann C, Schaible U, Schultze-Seemann W, Schollmeyer PJ: β-adrenergic, angiotensin II, and bradykinin receptors enhance neurotransmission in human kidney. *Hypertension* 1995;26:445–451.
31. Oakley R, Hudson C, Cruickshank R, Meyers D, Payne R, Rhem S, *et al*.: The cellular distribution of fluorescently labeled arrestins provides a robust, sensitive, and universal assay for screening G protein-coupled receptors. *Assay Drug Dev Technol* 2002;1:21–30.
32. Volovyk ZM, Wolf MJ, Prasad SV, Rockman HA: Agonist-stimulated beta-adrenergic receptor internalization requires dynamic cytoskeletal actin turnover. *J Biol Chem* 2006;281:9773–9780.
33. Szekeres PG, Koenig JA, Edwardson JM: The relationship between agonist intrinsic activity and the rate of endocytosis of muscarinic receptors in a human neuroblastoma cell line. *Mol Pharmacol* 1998;53:759–765.
34. Waelbroeck M: Activation of guanosine 5′-[γ-^{35}S]thiotriphosphate binding through M1 muscarinic receptors in transfected Chinese hamster ovary cell membranes: 2. Testing the "two-states" model of receptor activation. *Mol Pharmacol* 2001;59:886–893.
35. Bakker RA, Schoonus SBJ, Smit MJ, Timmerman H, Leurs R: Histamine H_1-receptor activation of nuclear factor-κ B: roles for G$\beta\gamma$- and G$\alpha_{q/11}$-subunits in constitutive and agonist-mediated signaling. *Mol Pharmacol* 2001;60: 1133–1142.
36. Mangel AW, Raymond JR, Fitz JG: Regulation of high-conductance anion channels by G proteins and 5-HT1A receptors in CHO cells. *Am J Physiol Renal Physiol* 1993; 264:F490–F495.
37. Pauwels PJ, Colpaert FC: Ca^{2k} responses in Chinese hamster ovary-K1 cells demonstrate an atypical pattern of ligand-induced 5-HT1A receptor activation. *J Pharmacol Exp Ther* 2003;307:608–614.
38. Della Rocca GJ, Mukhin YV, Garnovskaya MN, Daaka Y, Clark GJ, Luttrell LM, *et al*.: Serotonin 5-HT1A receptor-mediated Erk activation requires calcium/calmodulin-dependent receptor endocytosis. *J Biol Chem* 1999;274: 4749–4753.
39. Garnovskaya MN, Gettys TW, van Biesen T, Prpic V, Chuprun JK, Raymond JR: 5-HT1A receptor activates Na^+/H^+ exchange in CHO-K1 cells through $G_{i\alpha 2}$ and $G_{i\alpha 3}$. *J Biol Chem* 1997;272:7770–7776.
40. Demchyshyn L, Sunahara R, Miller K, Teitler M, Hoffman B, Kennedy J, *et al*.: A human serotonin 1D receptor variant (5HT1Dβ) encoded by an intronless gene on chromosome 6. *Proc Natl Acad Sci U S A* 1992; 89:5522–5526.
41. Ruat M, Traiffort E, Leurs R, Tardivel-Lacombe J, Diaz J, Arrang J, *et al*.: Molecular cloning, characterization, and localization of a high-affinity serotonin receptor (5-HT7) activating cAMP formation. *Proc Natl Acad Sci U S A* 1993;90:8547–8551.
42. Robert SJ, Zugaza JL, Fischmeister R, Gardier AM, Lezoualc'h F: The human serotonin 5-HT4 receptor regulates secretion of non-amyloidogenic precursor protein. *J Biol Chem* 2001;276:44881–44888.
43. Newman-Tancredi A, Cussac D, Quentric Y, Touzard M, Verriele L, Carpentier N, et al.: Differential actions of antiparkinson agents at multiple classes of monoaminergic receptor. III. Agonist and antagonist properties at serotonin, 5-HT1 and 5-HT2, receptor subtypes. *J Pharmacol Exp Ther* 2002;303:815–822.

Address reprint requests to:
Ryan McGuinness
MDS Sciex
1170 Veterans Boulevard, Suite 200
South San Francisco, CA 94080

E-mail: ryan.mcguinness@sciex.com

Ex. 2027
Axion v. Agilent
IPR2024-01467

Downloaded by 70.181.107.185 from www.liebertpub.com at 10/13/25. For personal use only.

Ex. 2027
Axion v. Agilent
IPR2024-01467