

Evaluating Cellular Impedance Assays for Detection of GPCR Pleiotropic Signaling and Functional Selectivity

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G-protein-coupled receptors can couple to different signal transduction pathways in different cell types (termed cell-specific signaling) and can activate different signaling pathways depending on the receptor conformation(s) stabilized by the activating ligand (functional selectivity). These concepts offer potential for developing pathway-specific drugs that increase efficacy and reduce side effects. Despite significant interest, functional selectivity has been difficult to exploit in drug discovery, in part due to the burden of multiple assays. Cellular impedance assays use an emerging technology that can qualitatively distinguish Gs, Gi/o, and Gq signaling in a single assay and is thereby suited for studying these pharmacological concepts. Cellular impedance confirmed cell-specific Gs and Gq coupling for the melanocortin-4 receptor and dual Gi and Gs signaling with the cannabinoid-1 (CB1) receptor. The balance of Gi versus Gs signaling depended on the cell line. In CB1-HEKs, Gi- and Gs-like responses combined to yield a novel impedance profile demonstrating the dynamic nature of these traces. Cell-specific signaling was observed with endogenous D1 receptor in U-2 cells and SK-N-MC cells, yet the pharmacological profile of partial and full agonists was similar in both cell lines. We conclude that the dynamic impedance profile encodes valuable relative signaling information and is sufficiently robust to help evaluate cell-specific signaling and functional selectivity. (*Journal of Biomolecular Screening* 2009:246-255)

Key words: GPCR, cellular impedance assays, functional selectivity, biased-agonism

INTRODUCTION

G-PROTEIN-COUPLED RECEPTOR (GPCR) SIGNALING is well known to be pleiotropic. A given receptor-agonist pair can activate different G-proteins depending on the cellular environment. Within a given cell type, different agonists can cause the same receptor to preferentially activate different downstream signaling pathways. This latter concept, variously referred to as functional selectivity, biased agonism, or ligand-directed trafficking, has generated intense interest in the drug discovery arena.¹⁻³ Conceivably, drugs would be tailored to selectively activate therapeutically relevant pathways rather than those associated with side effects. Translating this potential opportunity into practice has faced technical hurdles. Most traditional GPCR drug screening assays are specialized for individual G-protein classes thereby necessitating multiple

assays to evaluate differential signaling. New technologies that distinguish GPCR coupling in a single assay are needed to make functional selectivity screening a routine part of drug discovery.

New cell-based, label-free screening technologies have emerged that can detect GPCR coupling through each of the three main classes of G-proteins. Currently these include several cellular impedance-based platforms⁴⁻⁶ and a related dynamic mass redistribution platform.⁷ Each of these platforms uses unique proprietary algorithms to transform complex data into simple kinetic traces. Although absolute shape of the profiles varies as a result of different data transformation, in each case the response profiles are sensitive to activation of different G-protein pathways. One cellular impedance-based platform, CellKeyTM, is specifically tailored to GPCR detection and has the novel feature of producing response profiles that are consistent across a range of assay conditions and distinguish Gs, Gi/o, and Gq.

We recently evaluated cellular impedance assays for use in drug discovery focusing on direct detection of Gi-coupled receptors.⁸ The impedance data matched or exceeded traditional GiPCR assays in terms of the sensitivity and precision when testing agonists, antagonists, and positive modulators. In addition to these reported conclusions, which were based on

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Received Sep 30, 2008, and in revised form Oct 28, 2008. Accepted for publication Nov 14, 2008.

Journal of Biomolecular Screening 14(3); 2009
DOI: 10.1177/1087057108330115

endpoint measurements (net impedance change), we also gained experience with kinetic response profiles. Specifically, when impedance time course profiles revealed unexpected G-protein coupling, these findings were consistently confirmed with pathway-specific reagents. Thus, despite initial reservations regarding the qualitative nature of the profiles, these preliminary studies suggested that the kinetic impedance response could reveal useful information concerning divergent signaling pathways, which warranted further investigation as described herein. To focus on assay evaluation rather than biological discovery, receptors with well-established coupling to multiple G-protein transduction pathways were selected. Attempts were made to go beyond simply testing the manufacturer's G-protein-specific profiles and determine if these profiles are dynamic enough to evaluate complex GPCR pharmacology such as dual signaling and functional selectivity.

MATERIALS AND METHODS

Compounds

Dopamine (DA), dihydrexidine (DHX), SKF83959, SKF38393, A77636, SCH23390, acetylcholine, pertussis toxin (PTX), cytochalasin D, and U73122 were obtained from Sigma (St. Louis, MO). CP55940, Win55212-2, and O-2050 were purchased from Tocris Biosciences (Bristol, UK). MTIL, α -MSH, and SHU9119 were obtained from Phoenix Pharmaceuticals (Burlingame, CA).

Cell culture

The base media for CHO, HEK, SK-N-MC, and U-2 OS cells were Ham's F-12, DMEM, DMEM plus pyruvate and nonessential amino acids, and McCoy's 5A, respectively. Media were supplemented with 10% dialyzed FBS and 2 mM L-glutamine. CHO-K1 or HEK293 cells expressing human receptors as stable clones were cultured in appropriate selection medium (G418-sulfate or hygromycin B). Cell culture reagents were from MediaTech (Herndon, VA). Cells were harvested in Cellstripper™ and plated in 150 μ L in 96-well plates. Optimized cell seeding density per well for each cell type was as follows: 65 K for CHO, 50 K for HEK, 90 K for U-2, and 105 K for SK-N-MC. HEK cells were seeded 48 h before testing; all others were plated 24 h in advance.

CDS assays

Twenty-five minutes before agonist addition, cell culture media was replaced with 135 μ L of Hanks' balanced salt solution, 20 mM HEPES pH 7.4, 0.1% BSA (HHBS), and supplemented with antagonist as indicated. After incubating at 28 °C, plates were loaded in the CellKey™ (MDS Sciex, Concord, Ontario, Canada) and the baseline impedance (dZie) was established (5 min, 2-sec intervals). Agonist (15 μ L) was added from a 10 $\times$ stock solution to yield the final concentrations indicated in figure legends. Impedance was measured for the indicated times. Signal pathway-assessing reagents were included as follows: cytochalasin D (10 μ M) and U73122 (9 μ M) in HHBS for 30 min prior to agonist addition; PTX (100 ng/mL) in cell culture media 16 h prior to agonist addition.

Table 1. Potency and Efficacy Values of D1/5 Agonists

	<i>E_{max}</i> (% of DA)			<i>EC₅₀</i> (nM)		
	SK	U-2		SK	U-2	
DA	100	100		78 $\pm$ 0.2	16 $\pm$ 0.05	
SKF83959	24 $\pm$ 2	50 $\pm$ 5		1.0 $\pm$ 0.1	1.3 $\pm$ 0.04	
SKF38393	47 $\pm$ 8	83 $\pm$ 9		14 $\pm$ 0.1	11 $\pm$ 0.06	
DHX	96 $\pm$ 2	99 $\pm$ 4		11 $\pm$ 0.1	1.8 $\pm$ 0.1	
A77636	96 $\pm$ 6	96 $\pm$ 4		2.9 $\pm$ 0.04	0.054 $\pm$ 0.01	

Impedance data are the mean $\pm$ SEM of three independent experiments. DA, dopamine; DHX, dihydrexidine.

was established (5 min, 2-sec intervals). Agonist (15 μ L) was added from a 10 $\times$ stock solution to yield the final concentrations indicated in figure legends. Impedance was measured for the indicated times. Signal pathway-assessing reagents were included as follows: cytochalasin D (10 μ M) and U73122 (9 μ M) in HHBS for 30 min prior to agonist addition; PTX (100 ng/mL) in cell culture media 16 h prior to agonist addition.

Data analysis

All data represent extracellular impedance (dZie). Concentration response curves were generated by fitting data to a four-parameter logistic equation using Prism 4.0 software (GraphPad, San Diego, CA). Default data variance plotted in graphs and shown in the **Table** are SEM. Variance in text is standard deviation.

RESULTS

Gs, Gi/o, and Gq impedance profiles

To establish typical Gs, Gi/o, and Gq impedance response profiles, we selected receptors with well-defined coupling. Activating the Gs-coupled DA D5 receptor induced a decrease in cellular impedance (**Fig. 1**). Decreases in impedance appear to be unique to increases in cAMP and can be replicated by directly activating cAMP with forskolin (not shown). In contrast, activating the DA D2 receptor, a representative Gi-linked GPCR, resulted in a rapid increase in impedance. Pretreatment with the Gi-selective PTX eliminated the D2 response but not the D5 response (**Fig. 1**). The Gq-linked muscarinic acetylcholine receptor M1 also yielded an increase in impedance but only after a transient dip. As expected, the M1 response was not significantly affected by PTX. Although the transient dip in impedance is regarded as a hallmark of Gq-coupling responses, its magnitude was variable particularly at intermediate to low (<EC₅₀) concentrations of agonists. Thus, additional pathway characterization such as PTX sensitivity can help distinguish between Gq and Gi responses. These data

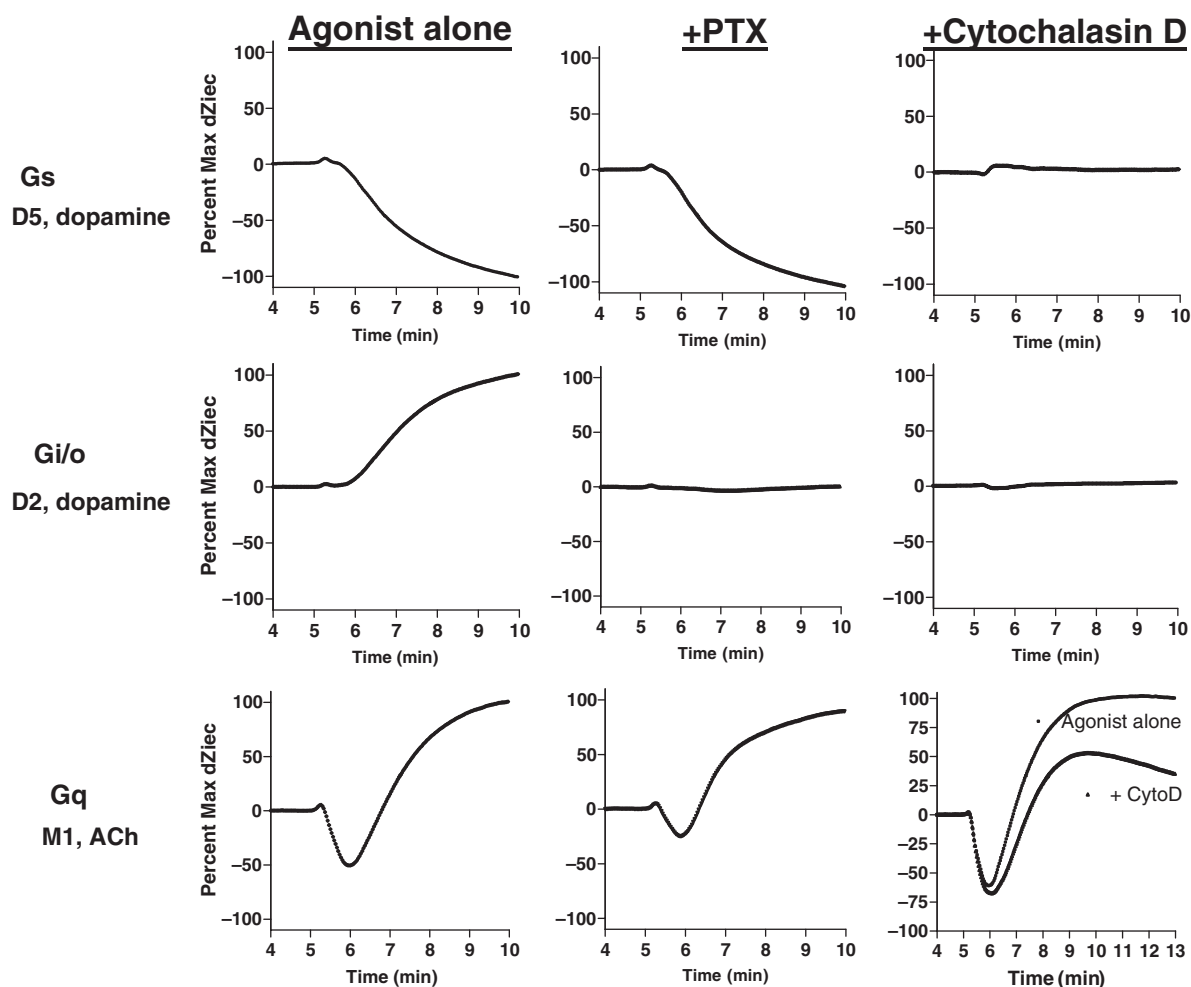


FIG. 1. Distinctive cellular impedance response profiles for Gs, Gi/o, and Gq signaling using CellKey™. The dopamine receptors D5 and D2 and the muscarinic M1 receptor were stimulated with agonist at concentrations (acetylcholine 10 nM, dopamine 1 nM for D2, 100 pM for D5) approximately 10-fold greater than the EC50. This concentration was used as the standard in control (left column), after pretreatment with pertussis toxin (middle), or after pretreatment with cytochalasin D (right). Untransfected CHO cells do not respond to dopamine or acetylcholine; stable CHO cell lines were used for each receptor. Ligands were added after a 5-min baseline was established.

are representative of experience with more than 20 assays in which response profiles matched second messenger literature data and/or were confirmed with pathway-specific inhibitors.

The mechanism by which impedance assays detect GPCR responses is not well studied. Pioneering experiments with cellular impedance focused on relating impedance changes to cellular morphology. These studies demonstrated exquisite sensitivity to changes in actin dynamics.^{4,9} Although GPCRs are known to alter actin dynamics,¹⁰⁻¹² the relationship between GPCR impedance responses and actin changes is untested. As shown in **Figure 1**, the inhibitor of actin polymerization, cytochalasin D, eliminated the impedance response to D5 and D2 receptor activation. In contrast, the M1 impedance response was only partially inhibited. In a similar CHO cell model, M1

activation has been shown to induce both spectrin reorganization as well as cytochalasin D-sensitive changes in actin cytoskeleton.¹³ It is unknown whether spectrin reorganization and partial cytochalasin D insensitivity is unique to M1-CHO or is common to Gq-coupled receptors. The present data indicate that impedance assays can detect GPCR-induced changes in the cytoskeleton that include but are not limited to modulation of actin.

Cell-specific signaling

Although the cellular environment is known to alter GPCR coupling, traditional GPCR assays are tailored to individual pathways thereby leading to an underappreciation of coupling

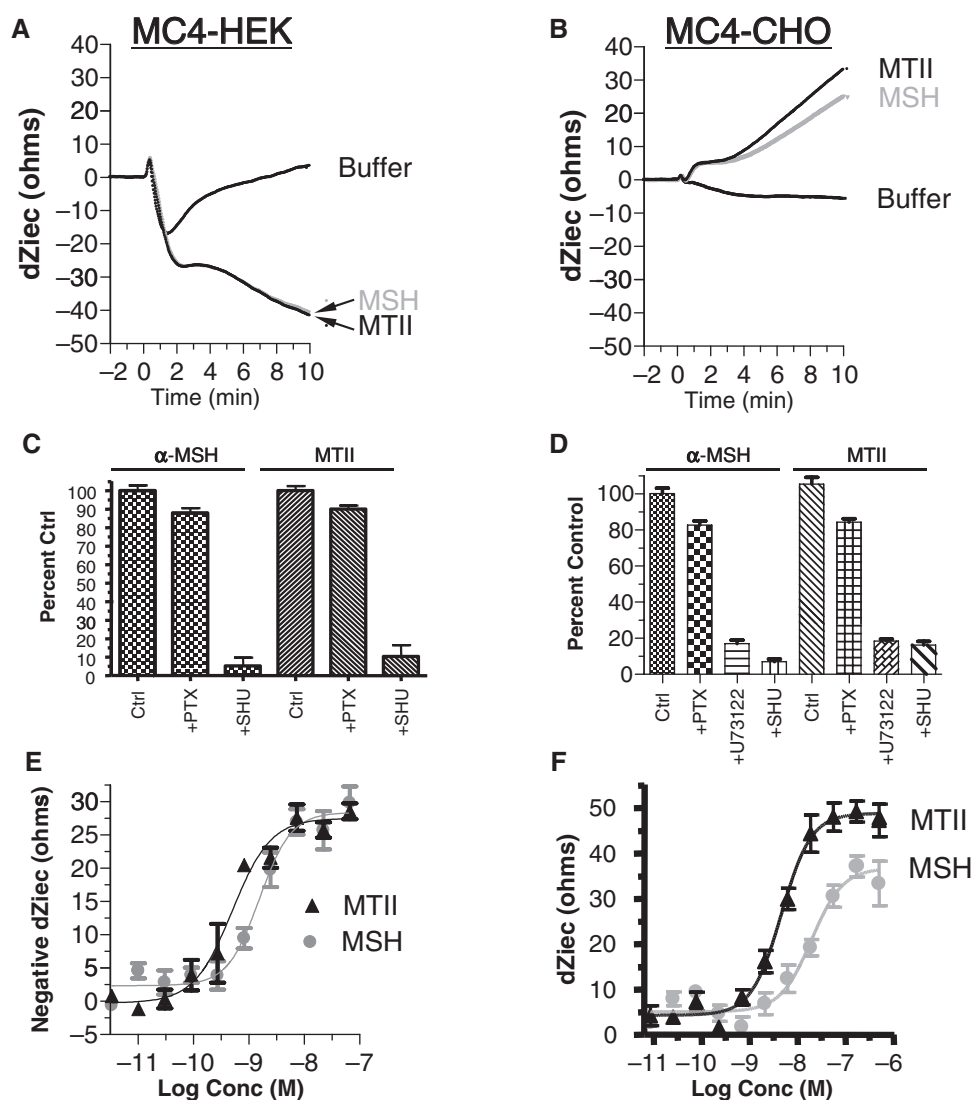


FIG. 2. Cell-type-specific G-protein coupling is readily detected by impedance profiles. (A) In HEK cells expressing the melanocortin-4 receptor (MC4R), the agonists α -MSH (50 nM) and MTII (50 nM) decreased impedance. (B) In MC4R-CHO cells, α -MSH (50 nM) and MTII (50 nM) increased impedance. The two agonists had similar response profiles at their respective EC₅₀ values. (C) In MC4R-HEK cells, these responses were specifically blocked by the MC4R-selective antagonist SHU9119 (1 μ M) but not by pertussis toxin (PTX; $n = 12$ wells). (D) In MC4R-CHO cells, the responses were specifically inhibited by SHU9119 (1 μ M) and the phospholipase C inhibitor U73122 (9 μ M), but not significantly by PTX ($n = 8$ wells). (E) Representative concentration response curves for α -MSH and MTII in MC4R-HEK cells ($n = 4$ wells) are shown. The pEC₅₀ values for α -MSH and MTII were 9.5 ± 0.4 and 9.1 ± 0.4 , respectively (≥ 3 experiments). (F) Representative concentration-response curves for α -MSH and MTII in MC4R-CHO cells ($n = 8$ wells) are shown. The pEC₅₀ values for α -MSH and MTII in MC4R-CHOs were 8.2 ± 0.3 and 8.0 ± 0.2 , respectively (≥ 3 experiments). The pIC₅₀ values for SHU9119 in HEKs and CHOs were 8.0 ± 0.1 and 8.0 ± 0.7 , respectively (4 experiments, α -MSH stimulus: 2 nM for HEKs and 50 nM for CHOs).

diversity. In contrast, impedance assays can detect activation of Gs, Gi, and Gq pathways and thus have the potential to readily detect cell-specific differences in G-protein coupling. For example, the melanocortin-4 receptor (MC4R) showed different coupling when expressed in HEK cells versus CHO cells. In MC4R-HEKs, the agonists α -MSH and MTII decreased

impedance (Fig. 2A), which matches the well-established Gs response for this receptor.¹⁴ In MC4R-CHO cells, both α -MSH and MTII increased impedance, which suggests either Gq or Gi/o coupling (Fig. 2B). Consistent with Gq coupling in CHO, the responses were blocked by pretreatment with the phospholipase C inhibitor U73122 whereas PTX had little

effect (**Fig. 2D**). Preferential coupling through Gq is not widely recognized but has been reported in GT1-1 neurons expressing endogenous MC4R.¹⁵

The Gs-like and Gq-like MC4R responses showed similar pharmacological characteristics. The MC4R antagonist SHU9119 inhibited both responses (**Fig. 2C, D**). MTII and α -MSH had similar relative potencies in HEK cells (pEC_{50} 9.5 ± 0.4 vs. 9.1 ± 0.4) and in CHO cells (pEC_{50} 8.2 ± 0.3 vs. 8.0 ± 0.2). The absolute agonist potency was significantly higher in the Gs-like MC4R-HEKs. Although this higher potency could be caused by more efficient coupling through Gs, we cannot rule out the possibility that impedance assays are more sensitive to coupling through Gs than Gq. Additional studies would be needed to assess the relative detection efficiency for each G-protein pathway. The greater efficacy of MTII versus α -MSH in the Gq-coupled MC4R-CHOs was consistently observed (**Fig. 2F**). This may be a cell-specific effect, because MTII has been reported as less efficacious than α -MSH in astrocytes and similar to α -MSH in HEK cells (**Fig. 2E**).

The shapes of the MC4R response profiles show subtle differences from the prototypical Gs and Gq profiles that are not uncommon and worth comment. The HEK cell response includes a fluid addition artifact that was clearly evident in both the buffer and the agonist traces. Fluid addition artifacts are more common with weakly adherent cell types like HEKs, but for unknown reasons, this effect was only modestly reduced by slower fluid addition in the MC4R-HEK cells. In the MC4R-CHO cells, the impedance profile exhibited a transient delay rather than the transient dip that typically precedes the increase in impedance associated with Gq signaling. Additional experiments are needed to investigate signaling processes associated with earliest phase of the Gq response profile.

Dual signaling profiles

The known diversity in impedance profiles for GPCR signaling is currently limited to the three standard Gs, Gi/o, and Gq traces⁵ (**Fig. 1**). If these profiles are simply nodes on a continuum rather than fixed responses, then receptors that activate multiple pathways should yield novel profiles. To test this idea, we selected the cannabinoid receptor (CB1), which can activate both Gi and Gs. In CB1-CHO cells, the selective agonists CP55940 and Win55212-2 produced Gi-like profiles. However, after inhibiting Gi signaling with PTX, both agonists induced Gs-like responses (**Fig. 3A, B**). No responses were detected in untransfected CHOs or after pretreatment with the CB1-selective antagonist O-2050, confirming a CB1-mediated response (data not shown). These impedance results are consistent with cAMP measurements in CB1-CHOs that demonstrated prominent Gi signaling that could be blocked with PTX to reveal a Gs response.^{16,17} In contrast, more

balanced Gs/Gi coupling has been observed for natively expressed CB1 in striatal and cortical neuronal cultures.^{17,18}

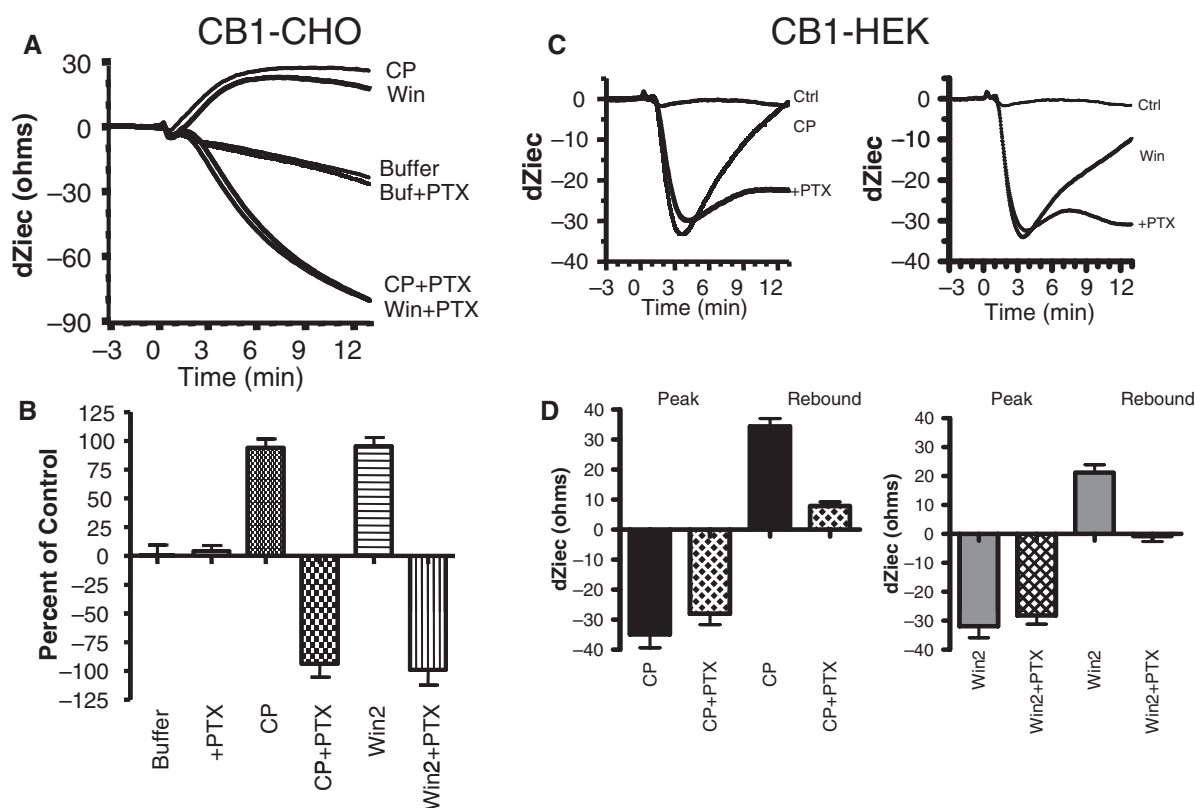
Impedance experiments with CB1 expressed in HEKs revealed a novel profile. Both CP55940 and Win55212-2 induced a rapid decrease in impedance followed by a robust reversal (**Fig. 3C**). Pretreatment with PTX did not alter the initial Gs-like impedance drop but dramatically reduced the Gi-like rebound (**Fig. 3C, D**). No responses were detected in untransfected HEKs or after pretreatment with the CB1 antagonist O-2050 (data not shown). This dual profile was not unique to CB1, because selective agonists for the dual Gs/Gi-coupled β_2 -adrenergic receptor produced indistinguishable biphasic profiles in A431 cells (M. Peters, unpublished data). This discovery of a novel dual-signaling profile indicates that the shape of impedance profiles encodes a qualitative readout of differential signaling.

Endogenous receptor signaling

The repertoire of signaling pathways activated by a particular GPCR is influenced by the expression and stoichiometry of receptor, G-proteins, and effector molecules. Because recombinant cell lines with high receptor expression can activate G-protein pathways not found under more physiological conditions,^{19,20} aspects of pleiotropic signaling were studied using native receptor complexes. The human U-2 osteosarcoma and SK-N-MC neuroblastoma cells express functional dopamine receptors that respond to D1/5 agonists.^{5,21} (Currently available ligands do not allow pharmacological distinction between D1 and D5 receptors.) In U-2 osteosarcoma cells, DA and the synthetic agonist DHX induced Gs-like responses⁵ (**Fig. 4A**). In contrast, the same ligands produced Gi/o-like profiles in SK-N-MC neuroblastoma cells (**Fig. 4B**). The Gi/o-like responses in SK-N-MCs were inhibited by PTX, whereas the Gs-like responses in U-2 cells were unaffected (**Fig. 4C, D**). Potency and efficacy values in both cell lines were highly similar to literature values²² and the responses were inhibited with the D1/5-selective antagonist SCH23390 (**Fig. 5C, D**).

Using D1-HEK cells, Ryman-Rasmussen et al.^{22,23} identified agonists with different pathway bias for stimulating adenylate cyclase and inducing receptor internalization. SKF83959 and SKF38393 were partial agonists at stimulating cAMP production, but did not induce D1 internalization. DHX and A77636 were full agonists in the adenylate cyclase assay and were super agonists in the internalization assay, inducing 110% and 118% of DA's response, respectively. The availability of ligands with different pathway biases provides a useful test set for evaluating impedance responses in cell lines that show distinct receptor coupling.

The synthetic ligands SKF83959, SKF38393, DHX, and A77636 induced qualitatively similar Gs-like impedance profiles in U-2 cells and Gi/o-like profiles in SK-N-MC cells (**Fig. 5A, B**, and data not shown). As expected, the impedance



response of each ligand was sensitive to PTX pretreatment in SK-N-MC cells, but was insensitive in U-2 cells (**Fig. 5B, D**). Thus, although the two cell lines show very different coupling biases by impedance measures, all the synthetic ligands used in this study reproduce the coupling profile seen with the native ligand DA. The ligands that reportedly are unable to induce receptor internalization had the same impedance profile shape as ligands that are super agonists for internalization.

In terms of quantitative impedance responses, SKF83959 and SKF38393 were partial agonists in both impedance assays, with SKF38393 being the more efficacious ligand (**Table**). This was also true in the adenylyl cyclase assay using D1-HEK cells.²² Both partial agonists had greater efficacy in U-2 cells than in SK-N-MC cells. The greater efficacy for a Gs-like response could reflect biased coupling by these ligands, but

could also be due to cellular or assay differences that are not understood. A77636 was a full agonist in both cell lines, but was 54-fold more potent in U-2 cells than in SK-N-MC cells. A potency difference of this magnitude was not seen with DA or the other three synthetic agonists. This result may reflect biased agonism for Gs over Gi/o by A77636 or more complex DA receptor expression in U-2 cells. Either way, the CellKey™ impedance profiles were robust with sufficient precision to enable studying pathway bias with endogenously expressed GPCRs.

DISCUSSION

Within drug discovery, the complexities of pleiotropic GPCR signaling have been underexploited as an opportunity and difficult to handle as a variable. Running multiple functional

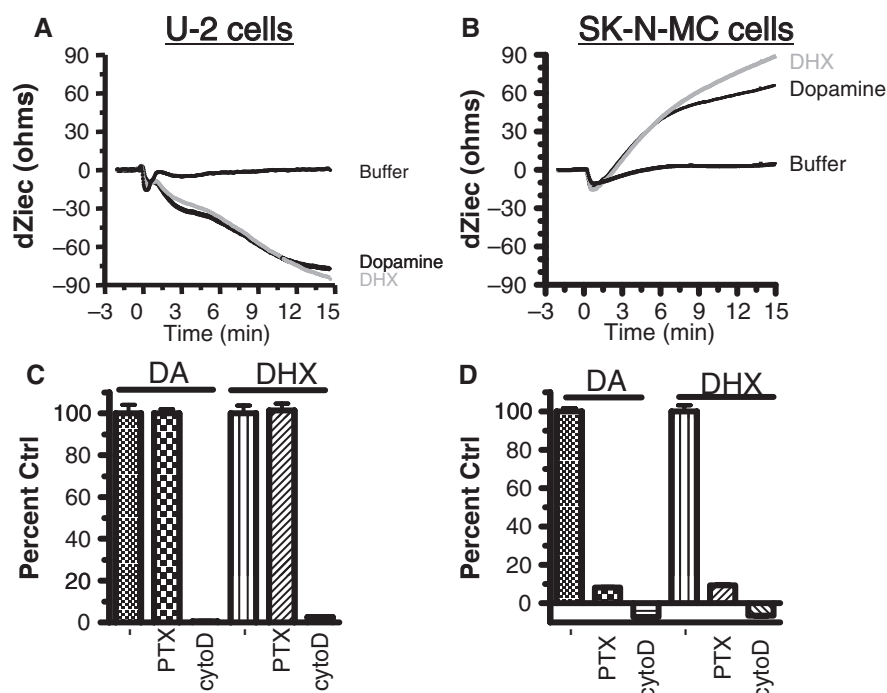


FIG. 4. Impedance response profile of endogenous D1/D5 reveals different coupling in U-2 and SK-N-MC cells. **(A)** Impedance response profile of human osteosarcoma U-2 cells treated with dopamine (DA; 1 μ M) or dihydrexidine (DHX; 80 nM). **(B)** Impedance response profile of human neuroblastoma SK-N-MC cells treated with DA (1 μ M) or DHX (80 nM). **(C)** U-2 cells were pretreated with cytochalasin D, pertussis toxin (PTX), or buffer, then treated with 300 nM DA or 30 nM DHX. **(D)** The SK-N-MC response to DA (1 μ M) and DHX (80 nM) was inhibited by pretreatment with either PTX or cytochalasin D ($n = 8$ wells).

assays requires substantial additional costs and resources. However, the recent development of instrumentation that can quantify cellular impedance in real time has provided inroads to detecting the classical Gs, Gi/o, and Gq pathways in a single assay. Although recent studies with impedance measurements have demonstrated the ability to distinguish each of these pathways using prototypic receptor-ligand pairs,⁵ the use of impedance profiles to discern cell-specific signaling, dual signaling, and functional selectivity has not been previously probed.

The cellular environment in which a GPCR is expressed can influence G-protein coupling.^{19,20} Although widely recognized, the scope and importance of cell-specific G-protein signaling has been difficult to assess with traditional assays. The present impedance data indicate that cell-specific signaling may be underappreciated and is not infrequent even in recombinant cell lines that use host cells containing functional Gs, Gi/o, and Gq pathways. For example, impedance data confirmed the widely accepted Gs coupling for the MC4R receptor in HEK cells,²⁴ whereas coupling through Gq was observed in CHO cells (Fig. 2A, B). Gq signaling appears to be biologically relevant based on studies with endogenously expressed MC4R in GT1 neurons.¹⁵ Analogous results were observed with the dual Gi/

Gs-coupled CB1. Impedance assays in CHO cells reveal a dominance of Gi coupling with Gs signaling detectable only after PTX treatment (Fig. 3A, B). This Gi coupling preference for CB1 in CHO cells matches published results.^{17,25} In contrast, impedance studies discovered a more balanced dual Gi/Gs-like profile in HEKs (Fig. 3C, D). Although not previously recognized, this CB1-HEK coupling compares favorably with data from native CB1 in neuronal cultures where a more substantial Gs signaling has been observed.^{17,18} Finally, endogenous D1/5 responses coupled through Gs and Gi in human osteosarcoma U-2 and neuroblastoma SK-N-MC cells, respectively (Figs. 4, 5). The present experiments do not address the mechanisms leading to the cell-specific signaling observed for D1/5, CB1, or MC4R. We cannot exclude the possibility that changes in receptor density and/or clonal selection may contribute to the signaling changes. Independent of mechanism, the data clearly illustrate the importance of evaluating cell-specific signaling during assay development to ensure that the desired signal transduction pathway is activated and to determine if other classical G-protein pathways are also affected.

A new response profile, clearly distinct from the standard Gs, Gi, or Gq profiles, was identified for CB1 in HEKs. The

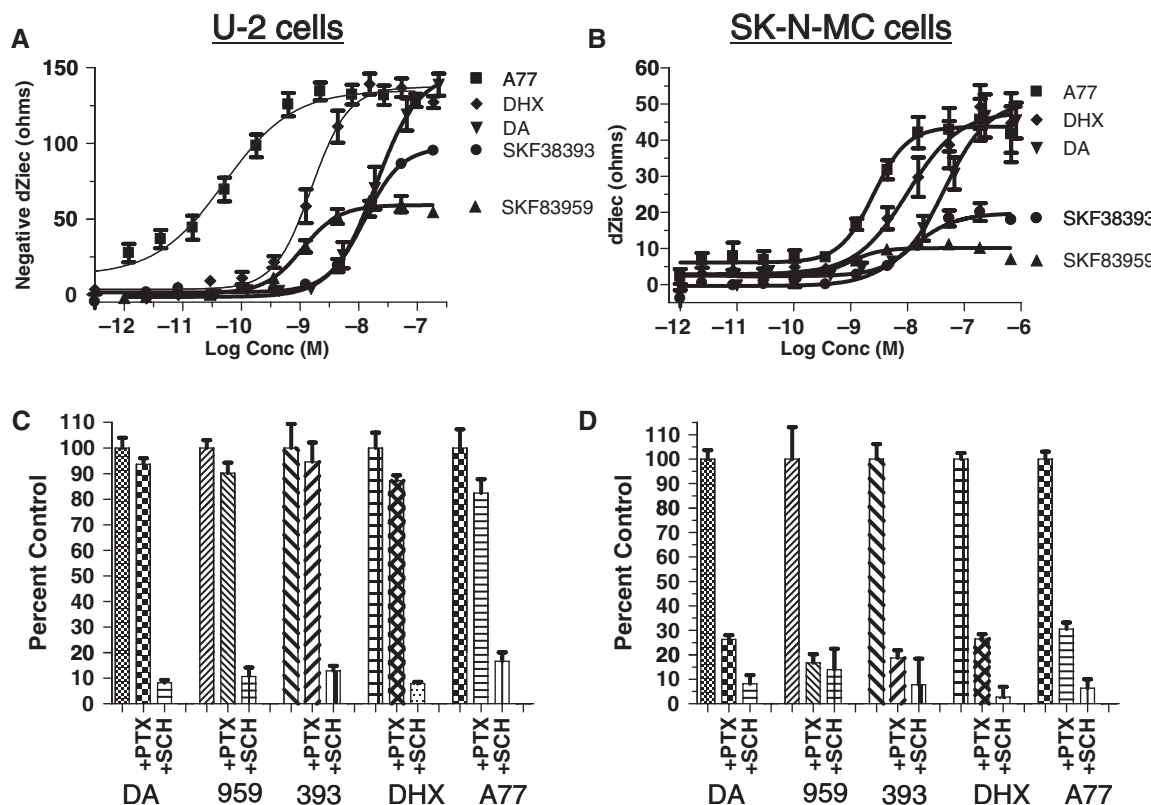


FIG. 5. Cellular impedance profiles of pathway-selective D1/5 agonists in differentially coupled native human cell lines. Dopamine (DA), dihydrexidine (DHX), A77636, and SKF38393 were tested for their impedance response efficacy and potency in both U-2 cells (A) and SK-N-MC cells (B) ($n = 4$ wells). Responses in U-2 cells (C) and SK-N-MC cells (D) showed similar inhibition to D1/5-selective antagonist SCH23959 (1 μ M) but differed in sensitivity to pertussis toxin.

shape of this profile and its selective alteration after PTX treatment match the dual Gs/Gi coupling expected for CB1. This profile was not limited to CB1; an indistinguishable trace was observed for a second Gs/Gi-coupled receptor (β -adrenergic). This dual profile (Fig. 3C) represents the first demonstration that impedance responses are dynamic in response to complex signaling. The dynamic quality of these profiles is significant because it suggests that the shape of impedance profiles may be used not only to discern dual signaling, but that changes in response shape may be used to detect ligands that preferentially modulate a particular pathway, that is, functionally selective ligands. The selective impact of PTX pretreatment on the rebound phase of the CB1-HEK impedance profile provides evidence consistent with this hypothesis. It should be noted that the present data only address qualitative comparisons of response profiles. The relative sensitivity of impedance assays to activation of different pathways is unknown. Thus, relative ratio of Gs:Gi activation observed in impedance assays may differ from other measures of these pathways. Despite this qualitative limitation, screening

for relative profile shape changes would be valuable in a drug discovery setting. For example, discovery of functionally selective CB1 agonists that favor either Gi or Gs would certainly be of interest in determining whether one can separate clinical efficacy from mechanism-based side effects typically associated with cannabinoids.

Among the emerging literature on functionally selective agonists, a set of well-validated D1 ligands are available as tools to evaluate the sensitivity of impedance assays to biased agonists. In studies using D1-HEK cells, synthetic ligands were identified that induced cAMP production, but not receptor internalization, and others that were super agonists for internalization.²² SK-N-MC cells endogenously express D1, but not D5 receptors,^{21,26} and therefore represent a useful cell line to compare the pharmacology between endogenously expressed/coupled D1 receptors to D1-transfected cells. In contrast to the results with D1-HEKs, the native ligand and four synthetic agonists all induced a Gi/o impedance response in SK-N-MC cells. Although Gi/o coupling has been reported,^{27,28} most literature indicates that D1 activates the Gs family of

signaling molecules²⁹ as observed with the U-2 cells. Compared with the native ligand, internalization-deficient ligands did not induce a different impedance phenotype. This was true in both cell lines; that is, impedance was not influenced by cell-specific signaling of the endogenous receptor. Some ligands showed quantitatively enhanced response in Gs-coupled U-2 cells compared with Gi/o-coupled SK-N-MC cells. This may reflect signaling bias although we cannot rule out the possibility that differences in D1/D5 subtype expression contribute to the enhanced response in U-2 cells. Nevertheless, the data clearly validate impedance assays as a tool with the novel ability to detect pathway-specific activity even for endogenously expressed receptors.

The molecular events in GPCR signaling detected by impedance assays are not well recognized. Early work pioneering the technology for cellular morphology assays revealed exquisite sensitivity capable of resolving 1-nm changes in cell diameter.⁹ These small changes in morphology were blocked by inhibitors of actin polymerization.³⁰ GPCRs are well known to induce actin-dependent changes in cellular morphology.¹⁰⁻¹² Specifically, cell spreading is associated with Gi activation^{31,32} whereas a rounded or stellate morphology is associated with Gs activation or cAMP elevation.³³⁻³⁵ More recently, actin polymerization has been shown to be required for the movement of GPCRs within the membrane associated with β -arrestin-mediated receptor recycling.^{36,37} Thus, not surprisingly, blocking actin polymerization inhibited or eliminated detection of GPCR responses by impedance assays (Fig. 1). To guide future experiments aimed at understanding the limitations of this technology, further mechanistic studies are needed to refine the signal pathways connecting GPCR activation with changes in actin dynamics.

Overall, we find that cellular impedance assays in combination with pathway-specific inhibitors are reliable for detecting and distinguishing G-protein coupling. The sensitivity to detect endogenous responses is straightforward to achieve and expands the scope of GPCR testing to include traditionally difficult issues such as native expression levels. The ability to identify changes in G-protein coupling in a single assay leads to significant benefits for drug discovery. These data demonstrate that impedance assays are highly robust for a wide range of GPCR applications and support broad use in SAR studies including functional selectivity.

ACKNOWLEDGMENTS

We thank Lois Ann Lazor, David Aharony, Reto Gadiant, and Jay Liu for their insightful comments on the manuscript.

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