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Label-free cell phenotypic assessment of the biased agonism and efficacy of agonists at the endogenous muscarinic M₃ receptors

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Abstract

Introduction—Efficacy describes the property of a ligand that enables the receptor to change its behavior towards the host cell, while biased agonism defines the ability of a ligand to differentially activate some of the vectorial pathways over others mediated through the receptor. However, little is known about the molecular basis defining the efficacy of ligands at G protein-coupled receptors. Here we characterize the biased agonism and cell phenotypic efficacy of seven agonists at the endogenous muscarinic M₃ receptors in six different cell lines including HT-29, PC-3, HeLa, SF268, CCRF-CEM and HCT-15 cells.

Methods—Quantitative real-time PCR and multiple label-free whole cell dynamic mass redistribution (DMR) assays were used to determine the functional muscarinic receptors in each cell line. DMR pathway deconvolution assay was used to determine the pathway biased activity of the muscarinic agonists. Operational agonism model was used to quantify the pathway bias, while macro-kinetic data reported in literature was used to analyze the biochemical mechanism of action of these agonists.

Results—Quantitative real-time PCR and ligand pharmacology studies showed that all the native cell lines endogenously express functional M₃ receptors. Furthermore, different agonists triggered distinct DMR signals in a specific cell line as well as in different cell lines. DMR pathway deconvolution using known G protein modulators revealed that the M₃ receptor in all the six cell lines signals through multiple G protein-mediated pathways, and certain agonists display biased agonism in a cell line-dependent manner. The whole cell efficacy and potency of these agonists were found to be sensitive to the assay time as well as the cell background. Correlation analysis suggested that the whole cell efficacy of agonists is correlated well with their macro-dissociation rate constants.

Discussion—This study implicates that the endogenous M₃ receptors are coupled to multiple pathways, and the muscarinic agonists can display distinct biased agonism and whole cell phenotypic efficacy.

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Appendix A. Supplementary data Supporting Information Available: Table S1 summarizes the EC₅₀, τ , and relative maxima values of all muscarinic receptor agonists tested. Fig. S1 shows the relative maxima of all seven agonists in different cell lines. Fig. S2 to S6 show the pathway deconvolution results in HT-29, HeLa, SF268, PC3 and CCRF-CEM cells, respectively. Fig. S7 shows the dose-dependent early DMR responses of all agonists in all six cell lines. This information is available free of charge via the Internet at <http://www.sciencedirect.com/science/journal/10568719>. Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vascn.2013.07.005>.

Keywords

Biased agonism; Drug residence time; Dynamic mass redistribution; G protein-coupled receptor; Efficacy; Muscarinic M₃ receptor

1. Introduction

Efficacy and affinity, the two hallmarks of drug pharmacology, are considered to be totally independent properties of drugs (Onaran & Costa, 2012). Affinity is the ability of the drug to bind to a receptor, while efficacy is the ability of the drug to change the behavior of the receptor towards the host cell. The concept of 'efficacy' has evolved over the past decades, owing to the increasing resolution of pharmacological assays to characterize the drug pharmacology. This is the best exemplified by drugs at G protein-coupled receptors (GPCRs), a family of receptors that are the master regulator of a wide range of physiological processes of cells and tissues (Neves, Ram, & Iyengar, 2002). As a family of allosteric proteins designed to transmit information GPCRs can display rich behaviors, ranging from pleiotropic coupling with multiple G proteins to receptor internalization, oligomerization, desensitization, and interaction with distinct membrane guest proteins (Kenakin & Miller, 2010). As a result, efficacy becomes vectorial (positive and negative cell activation) and pluridimensional (assay readout-dependent), instead of being linear in controlling different receptor behaviors (Galandrin & Bouvier, 2006; Kenakin, 2005, 2009). To date, the molecular mechanism of efficacy is mostly explored in terms of 'active state(s)', a conformation that triggers cellular response or a change in the behavior of the receptor towards the host cell (Kahsai et al., 2011; Liu, Horst, Katritch, Stevens, & Wuthrich, 2012; Sauliere et al., 2012).

In the recent years, amassing data suggest that label-free biosensor-enabled dynamic mass redistribution (DMR) assay is capable of translating an agonist-activated signal transduction process into a real-time whole cell phenotypic response under non-equilibrium condition (Fang, 2011). The rapid onset DMR responses of many agonists for almost all GPCRs tested to date suggest that receptor signaling proceeds right after agonist binding but long before reaching equilibrium binding (Fang, Li, & Ferrie, 2007); and some, but not all, of signaling events downstream the activated receptor can continue propagating after agonist removal (Goral, Jin et al., 2011; Goral, Wu, Sun, & Fang, 2011). Furthermore, the ability of several antagonists to block endogenous M₃ receptor signaling in HT-29 cells has been recently found to correlate with their residence time (the reciprocal of K_{off}) (Deng, Wang, Su, & Fang, 2012). These studies suggest the important role of drug residence time in GPCR signaling, and thus ligand efficacy. We herein characterize the signaling pathways of the endogenous M₃ receptors in six different cell lines, and deconvolute the biased agonism and cell phenotypic efficacy of seven agonists in these cell lines (Fig. 1). We found that these agonists displayed time- and cell line-dependent efficacy and potency; and their whole cell phenotypic efficacy is correlated well with their macro-dissociation rate constants.

2. Materials and methods

2.1. Materials

Atropine, bethanechol chloride, cholera toxin (CTx), methacholine chloride, and pertussis toxin (PTx) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acetylcholine chloride, carbachol (carbamoylcholine chloride), gallamine triethiodide, ipratropium bromide, McN-A-343, oxotremorine sesquifumarate, oxotremorine M, and pilocarpine hydrochloride were obtained from Tocris Bioscience Co. (St. Louis, MO, USA). Tiotropium was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). G_{oq} inhibiting

component (QIC), a $G_{\alpha q}$ -specific inhibitor structurally related to YM-254890 (Schröder et al., 2010), was obtained from Prof. Evi Kostenis (University of Bonn). Except for QIC which was stocked in 50 mM, all other compounds were stocked in 100 mM. Acetylcholine was dissolved in water, while all other drugs were prepared in dimethyl sulfoxide (DMSO). Before use, all compounds were freshly diluted using the assay buffer (1 times Hank's balanced salt solution (HBSS) buffer, 20 mM Hepes, pH 7.1) to the indicated concentrations. Both Epic® 384-well biosensor cell culture compatible and fibronectin-coated microplates were obtained from Corning Incorporated (Corning, NY, USA).

2.2. Cell culture

All six cell lines were obtained from American Type Cell Culture (Manassas, VA, USA). They are human colorectal adenocarcinoma cell line HT-29, human colorectal carcinoma cell line HCT-15, human epitheloid cervix carcinoma cell line HeLa, human glioblastoma cell line SF268, human prostate cancer cell line PC3, and human T cell lymphoblast-like cell line CCRF-CEM. Except for CCRF-CEM which is a suspension cell line, the rest are anchorage dependent. These cells were passaged at 37 °C with 5% CO₂ using the following complete medium: McCoy's 5A medium for HT-29, Eagle's Minimum Essential Medium for HeLa, F-12K Medium for PC3, and RPMI 1640 medium for HCT-15, SF268 and CCRF-CEM. All media were supplemented with 10% fetal bovine serum, 4.5 g/l glucose, 2 mM glutamine, 100 µg/ml penicillin and streptomycin. All anchorage-dependent cells were passed with trypsin/ethylene-diaminetetraacetic acid when approaching 90% confluence to provide new maintenance culture on T-75 flasks and experimental culture on the biosensor microplates. For DMR assays, both the cell seeding density and biosensor surface were optimized for each cell line so that all cells reached ~95% confluency after overnight culture (~20 h). The fibronectin coated biosensor plates were used for HCT-15, SF268 and CCRF-CEM cells, while the cell culture compatible biosensor plates were used for the remaining cell lines. The optimal seeding densities were found to be 30 K, 32 K, 25 K, 15 K, 20 K and 40 K per well for HCT-15, HT-29, HeLa, SF268, PC3 and CCRF-CEM cells, respectively. The optimal seeding densities were 25 K, 15 K, 25 K, 25 K, and 21 K for A431, A498, A549, DU145, and HepG2 cells, respectively.

2.3. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from the respective cell line using an RNeasy mini kit (Qiagen, Cat#74104). On-column DNase digestion was performed using RNase-free DNase set (Qiagen, Cat#79254) to eliminate genomic DNA contamination. The concentration and quality of total RNA were determined using a Nanodrop 8000 (Thermo Scientific). Customized PCR-array plates for 352 G protein-coupled receptor (GPCR) genes and reagents were ordered from SABiosciences (Qiagen, Valencia, CA). About 1 µg total RNA was used for each 96-well PCR-array. The PCR-array was performed on an ABI 7300 Real-Time PCR System following the manufacturer's instructions.

2.4. DMR assays

All DMR assays were performed in microplate under the persistent stimulation condition, where cells are exposed to a compound throughout the assays (Deng et al., 2012). These assays were performed using conventional Epic® system (Corning Incorporated), which is a wavelength interrogation reader system tailored for resonant waveguide grating (RWG) biosensors in microplates (Fang, Ferrie, Fontaine, Mauro, & Balakrishnan, 2006; Li, Ferrie, & Fang, 2006). This system consists of a temperature-control unit, an optical detection unit, and an on-board liquid handling unit operated by robotics. The detection unit is centered on integrated fiber optics, and enables kinetic measures of cellular responses with a time interval of about 15 s. For toxin treatment, cells were pretreated with 100 ng ml⁻¹ PTx for overnight, or 400 ng ml⁻¹ CTx for 3 h, or 1 µM QIC for 3 h, all in the complete medium at

37 °C/5% CO₂. Afterwards, the confluent cells were washed twice and maintained with the assay buffer (HBSS) and then incubated within the reader system for about 1 h. Antagonist pretreatment was performed in HBSS for 1 h. After a 2-min baseline was established, agonist solutions were transferred into the biosensor wells using the on-board liquid handling device, and the cell responses were recorded in real time. All studies were carried out with duplicates and two independent measurements (four replicates in total) unless specifically mentioned.

2.5. Data analysis

All experimental data were analyzed by using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA). The EC₅₀ values and maxima were obtained by fitting the dose DMR response curves with nonlinear regression. The relative maxima of each ligand were obtained by normalizing to the maxima of acetylcholine in the respective cell line. One-way ANOVA followed by Tukey's multiple comparison test was used to determine the significance of the difference among the DMR responses of different agonists in a respective cell line.

3. Results

3.1. Expression patterns of endogenous muscarinic receptors in six native cell lines

For label-free cell phenotypic profiling of a panel of seven muscarinic receptor agonists, we first examined the expression patterns of endogenous muscarinic receptors across 25 different cell lines using quantitative RT-PCR. Results showed that only six cell lines endogenously express predominantly M₃ receptors at the mRNA level (Table 1). These cell lines were the colon cancer cell lines HT-29 and HCT-15, the prostate cancer cell line PC3, the epitheloid cervix carcinoma cell line HeLa, the glioblastoma cell line SF268, and the T cell leukemia cell line CCRF-CEM. Using three control genes as controls, the relative mRNA level for the M₃ receptors was found to be the lowest in HeLa and SF268 cells and the highest in PC3 cells.

3.2. Characterization of functional muscarinic receptor(s) in the six cell lines

Next, we determined the functional muscarinic receptor subtype(s) in these cell lines using multiple DMR assays. First, DMR agonism assay showed that 16 μM acetylcholine, the non-selective muscarinic receptor native agonist, triggered detectable DMR but with distinct characteristics in the six cell lines (Fig. 2a). In contrast, McN-A-343, a M₁- and M₄-selective bitopic orthosteric/allosteric partial agonist (Mitchelson, 2012; Valant et al., 2008), up to 64 μM triggered negligible DMR, identical to the negative control (buffer), in the six cell lines (Fig. 2b), suggesting that there is no or little DMR response originating from endogenous M₁ or M₄ receptors in all the six cell lines. Second, DMR antagonist assay showed that gallamine, a M₂-selective allosteric antagonist (Huang, Prilla, Mohr, & Ellis, 2005), up to 64 μM only slightly suppressed the early signal (i.e., decreased the kinetics) of the DMR of 4 μM acetylcholine in HCT-15 cells, but not any other cell lines including SF268 (Fig. 2c), suggesting that there is no or little DMR response originating from endogenous M₂ receptors in all the six cell lines. In contrast, the three muscarinic antagonists, atropine, ipratropium, and tiotropium, all dose-dependently blocked the DMR of 4 μM acetylcholine in HCT-15 cells (Fig. 2d), and gave rise to a similar potency rank order across the six cell lines (Table 2). Although these antagonists are non-selective (Casarosa et al., 2009), their potency rank order and inhibition profiles obtained suggest that the acetylcholine DMR is mostly originated from a single muscarinic receptor subtype in all the six cell lines. This is further confirmed by the comparable potency rank order for the panel of seven agonists to desensitize or block the DMR arising from subsequent stimulation with 4 μM acetylcholine (Table 2). Third, DMR pathway deconvolution data showed that

the acetylcholine DMR signals in all the six cell lines were primarily resulted from the $G_{\alpha q}$ pathway (see below). Fourth, agonist profiling of the five M_3 -negative control cells including A431, A498, A549, Du145 and HepG2 showed that neither 10 μ M acetylcholine nor 10 μ M oxotremorine M triggered any detectable DMR, suggesting that the DMR assays appear to be only sensitive to endogenous receptors with high or moderate expression levels. Together, these results suggest that the endogenous M_3 receptors account primarily for the DMR signals obtained in these cell lines. This was further confirmed by literature mining showing that HT-29 (Kopp et al., 1989; Yang & Frucht, 2000) and PC-3 (Luthin, Wang, Zhou, Dhanasekaran, & Ruggieri, 1997) almost exclusively express the M_3 receptor subtype, while HeLa (Nginamau, Maehle, & Jonsson, 2011) and CCRF-CEM (Yoshikawa, Fujii, & Kawashima, 2005) are known to express functional M_3 receptors.

3.3. DMR characteristics of the muscarinic receptor agonists

Next, we compared the DMR characteristics of all seven agonists, each at its $1 \times EC_{100}$, in the six different cell lines. The characteristic DMR of these agonists was summarized in Fig. 3. Several interesting features emerged. First, pilocarpine only displayed weak agonistic activity in HCT-15, PC3 and CCRF-CEM, the three cell lines that have relatively high expression of the M_3 receptors at the mRNA level. In contrast, oxotremorine was active in all cell lines except for HeLa, while the remaining agonists were active in all cell lines. Given that the agonistic activity of partial agonists is more sensitive to the receptor expression level (Charlton, 2009), these results are largely in agreement with the mRNA expression level of the M_3 receptors in the respective cell line. Second, the maximal DMR signals of different agonists, when being active, shared similar characteristics in a specific cell line; however, a ligand resulted in clearly distinct DMR across different cell lines. Third, except for PC3 cells, the DMR of all agonists, when being active in a specific cell line, generally remains elevated over the assay period (~ 1 h). Fourth, different agonists displayed a cell line-dependent efficacy. Compared to the maximal responses of acetylcholine, oxotremorine M displayed noticeably higher efficacy in CCRF-CEM, SF268 and HeLa cells, while carbachol gave rise to noticeably lower efficacy in HeLa cells, and methacholine had relatively lower efficacy in PC3 and HeLa cells (Fig. 3, Supplementary Fig. S1). Furthermore, for the three partial agonists including bethanechol, oxotremorine and pilocarpine, all displayed relatively higher efficacy in CCRF-CEM, compared to the other cell lines (Fig. 3, Supplementary Fig. S1). Together, these results suggest that different muscarinic receptor agonists display cell line-dependent potency and efficacy.

3.4. Distinct time-dependent potency of different muscarinic receptor agonists

Next, we analyzed the time dependent potency of different muscarinic receptor agonists only in HTC-15, HT-29, PC3 and CCRF-CEM cells, given that their DMR signals are robust in these cell lines, but not in HeLa and SF268 cells. Nonlinear regression analysis gave rise to a series of time-dependent EC_{50} values, each at specific time-point post stimulation, for agonists that were active in the respective cell line (Fig. 4). Results showed that different ligands exhibited distinct time-dependent potency changes in different cell lines. In HCT-15 cells carbachol exhibited a relatively small increase in potency over time, while metacholine, acetylcholine and oxotremorine M displayed an initial small decrease in potency, followed by a small increase in potency over time (Fig. 4a). In contrast, the potency of bethanechol showed little change over time, but oxotremorine gave rise to the biggest change in time-dependent potency (Fig. 4b). For the other three cell lines, the potency of carbachol, oxotremorine M, metacholine and acetylcholine generally decreased over time (Fig. 4c, e, and g), while the potency of both bethanechol and oxotremorine exhibited little sensitivity over time in HT-29 or CCRF-CEM cells (Fig. 4d and f, respectively), but displayed an initial increase followed by a decrease in PC3 cells (Fig. 4h).

3.5. Cell line-dependent biased agonism of muscarinic agonists

We next examined the biased agonism of muscarinic receptor agonists using the established pathway deconvolution approach (Schröder et al., 2010; Verrier et al., 2011). Here, CTx was used to cause the permanent activation of $G_{\alpha s}$ by ADP ribosylation of an arginine residue of the protein, while PTx was used to result in the permanent inhibition of $G_{\alpha i}$ by ADP ribosylation of a cysteine of the protein, and QIC to selectively inhibit $G_{\alpha q}$ protein. These toxins were used to pretreat the cells before stimulation with a specific muscarinic receptor agonist. Results showed that in HCT-15 cells the acetylcholine DMR was suppressed partially by CTx, or mostly by QIC, or completely by the combination of CTx and QIC, while PTx only slightly slowed down the kinetics of the acetylcholine DMR (Fig. 5a), suggesting that the acetylcholine activated M_3 receptors in HCT-15 cells propagate signaling through both $G_{\alpha q}$ and $G_{\alpha s}$ pathways. Almost identical trend was found for oxotremorine M (Fig. 5b), carbachol (Fig. 5c), and methacholine (Fig. 5d), and to less extent, bethanechol (Fig. 5e). The DMR of oxotremorine or pilocarpine was originated only from the $G_{\alpha q}$ pathway, since QIC alone completely abolished their DMR (Fig. 5f and g, respectively). Interestingly, one-way ANOVA followed by Tukey's multiple comparison test suggests that the remaining DMR after QIC pretreatment gave rise to an order in DMR amplitude of acetylcholine ~ oxotremorine M > carbachol ~ methacholine $\gg$ bethanechol (Fig. 5h), suggesting that these agonists have distinct efficacy to activate the $G_{\alpha s}$ pathway. These results suggest that oxotremorine and pilocarpine are two partial agonists for the $G_{\alpha q}$ pathway, but not the $G_{\alpha s}$ pathway in HCT-15 cells, while the other agonists activate both pathways.

The pathway deconvolution also showed that in HT-29 cells QIC alone almost completely inhibited the DMR arising from all seven agonists (Supplementary Fig. S2). Neither CTx nor PTx had obvious effect on the DMR of all agonists. Combining QIC with PTx and/or CTx made the cells responding to all agonists with a small, rapid, but detectable DMR, similar to the treatment with QIC alone. These results suggest that the activation of endogenous M_3 receptors in HT-29 results in signaling primarily through the $G_{\alpha q}$ pathway, and also to small degree a non- $G_{\alpha s}$, $G_{\alpha q}$, $G_{\alpha i}$ pathway (possibly G12/13 pathway).

In HeLa cells the activation of the M_3 receptors also primarily mediated signaling via the $G_{\alpha q}$ pathway, as evidenced by the significant attenuation of the DMR signals of the five active agonists by the QIC treatment (Supplementary Fig. S3). Both PTx and CTx also altered the late DMR responses of these agonists. Only the combination of PTx, CTx and QIC, but not QIC with CTx or PTx, completely blocked the DMR of these five agonists. Together, these results suggest that the activation of endogenous M_3 receptors in HeLa may mediate signaling via multiple pathways.

In SF268 cells QIC greatly suppressed the DMR of all six active agonists, while PTx partially suppressed the DMR of these agonists (Supplementary Fig. S4). The combination of QIC and PTx completely abolished the DMR of these agonists. These results suggest that the activation of endogenous M_3 receptors in SF268 may mediate signaling via both the $G_{\alpha i}$ and $G_{\alpha q}$ pathways.

In PC-3 cells, QIC, PTx, and CTx all had obvious effects on the DMR of all six active agonists (Supplementary Fig. S5). The combination of QIC with PTx and CTx only completely blocked the acetylcholine DMR, but not the DMR of the other five agonists. These results suggest that the activation of endogenous M_3 receptors in PC-3 may mediate signaling via multiple pathways. Similar results were also observed in CCRF-CEM cells (Supplementary Fig. S6).

Collectively, these results suggest that the endogenous muscarinic M₃ receptor can mediate signaling via multiple pathways including G_{αq}, G_{αi}, G_{αs}, and possibly G_{12/13} pathways; specific pathways contributing to the DMR are cell background dependent; and muscarinic agonists can display biased agonism in a cellular context dependent manner.

3.6. Label-free cell phenotypic potency and efficacy of muscarinic receptor agonists

Given the complexity in signaling pathways contributing to the DMR as well as the time-dependent potency observed in the four cell lines, we chose two time points, one at early and another at 50 min post stimulation, to determine the potency and efficacy of all ligands in the six cell lines. The early time points were 15 min, 2 min, 3 min, 10 min, 3 min and 3 min post stimulation for HCT-15, HT-29, PC3, CCRF-CEM, SF268 and HeLa cells, respectively. The potency and efficacy for all agonists in the six cell lines were obtained using nonlinear regression and summarized in Supplementary Table S1. The early DMR dose responses were summarized in Supplementary Fig. S7, while the DMR amplitudes at 50 min post stimulation were shown in Fig. 6. Results showed that both the potency and efficacy of agonists are time- and cell line-dependent.

3.7. Operational agonism analysis

The Black and Leff model of operational agonism (Black & Leff, 1983; Kenakin, 2009) defines the value of τ , the ratio of receptor density to the efficiency of signal transduction by the agonist–receptor complex, as

$$\text{Response} = \frac{[A]^n \tau^n E_{\max}}{[A]^n \tau^n + ([A] + K_A)^n} \quad (1)$$

where $[A]$ is the agonist concentration, $E_{\max}$ the maximal response capability of the receptor in the respective cell line, K_A the equilibrium dissociation constant of the agonist–receptor complex, n the slope of the dose DMR response curve, and τ the efficacy equal to the ratio of receptor density to the efficiency of signal transduction by the ligand–receptor complex.

Given the relatively low expression levels of endogenous M₃ receptors in the six cell lines it is obviously difficult to directly determine the binding affinity of all agonists. Further complication arises from the observations that the binding affinity of a ligand to a GPCR is often cell line dependent and sensitive to the co-existence and nature of the receptor-interacting signaling protein (Kenakin, 2013). Thus, we first estimated the K_A value of each agonist in the respective cell line by fitting its dose DMR response curve with the Eq. (1), given (Kenakin, 2013):

$$K_A = EC_{50} \left((2 + \tau^n)^{\frac{1}{n}} - 1 \right). \quad (2)$$

Analysis of the dose responses of five agonists that triggered robust DMR in HCT-15 or HT-29 showed that the estimated pK_A values (Table 3) are somewhat different from, but generally tracked the rank order with, those obtained using radiobinding assays with CHO-M₃ cell lines (Sykes, Dowling, & Charlton, 2009). The reported pK_i values were -4.87 , -4.09 , -4.52 , -4.61 , and -3.71 for acetylcholine, carbachol, methacholine, oxotremorine M, and bethanechol, respectively. Furthermore, the operational bias analysis suggests that among the five agonists that resulted in robust DMR in HCT-15 cells, only bethanechol is clearly biased towards the early response. In contrast, bethanechol was found to be biased towards the late G_{αq}-mediated response in HT-29, compared to the early response mediated through both G_{αq}- and possibly G_{12/13}-pathways (Supplementary Fig. S2).

3.8. The biochemical mechanism of action of muscarinic receptor agonists

Lastly, to estimate the biochemical mechanism of action of these agonists, we used their known K_i , K_{on} and K_{off} values which were obtained using radioligand binding assays (Sykes et al., 2009). The biochemical mechanism of action defines the interaction between a drug and its target that creates a binding kinetics-determined response (Fang, 2013; Swinney, 2004). Given that in practice it is obviously difficult to accurately determine the binding kinetics of each ligand for each receptor-G protein complex in each cell line, as well as our operational model analysis pointed to the similarity between the K_A values calculated (Table 3) and those measured using radioligand binding assays (Sykes et al., 2009), we felt that it is possible to estimate the biochemical mechanism of action of muscarinic receptor agonists using the reported macro- K_i values and macro-binding kinetics. The M_3 receptor is known to couple with multiple G proteins in a specific cell line (Caulfield & Birdsall, 1998; Jakubik, Janickova, Randakova, Ei-Fakahany, & Dolezal, 2011), and certain fractions of M_3 receptor in a specific cell line can be precoupled with specific G protein(s), in particular G_{aq} proteins (Jakubik et al., 2011; Qin, Dong, Wu, & Lambert, 2011), as predicted by ensemble theory (Boehr, Nussinov, & Wright, 2009; Fang, 2012). Furthermore, the binding characteristics of ligands, in particular agonists, are sensitive to the presence and nature of receptor interacting proteins (Kenakin, 2013).

We examined the agonist responses in HCT-15 cells, since all the seven ligands were active in this cell line but with a wide range of efficacy (Fig. 3a). First, correlation analysis showed that for all seven agonists tested their EC_{50} values at 15 min post stimulation were found to be linear with the corresponding EC_{50} values at 50 min post stimulation (Fig. 7a), suggesting that both early and late DMR were resulted from the activation of the same receptor. Second, the relative maxima at 15 min were deviated from the linear relation with their corresponding relative maxima at 50 min (Fig. 7b). This suggests that there are two different cellular events/signaling pathways contributing differently to the early and late DMR responses, respectively; and the coupling efficiency to the pathway contributing to the late response was relatively stronger than that dominating the early response (Onaran & Costa, 2012) (Fig. 7c). This was confirmed by the pathway deconvolution results showing that the G_{aq} -pathway contributes to the early response, while both G_{aq} - and G_{as} -pathways co-contribute to the late response (Fig. 5). Third, the efficacy τ values at 15 min were found to be in linear with those at 50 min, regardless whether the τ values were estimated based on the reported K_i or the calculated K_A (Fig. 7d). Interestingly, the correlation analysis between the τ values at 15 min and 50 min obtained using the reported K_i values gave rise to a slope one-fold greater than that obtained using the calculated K_A values. These results suggest that although there is a system bias towards the late over the early responses, most of the agonists examined displayed little signaling bias (Table 3). This was confirmed by the pathway deconvolution results (Fig. 5), and further confirmed by the linear relation between the $\log(\tau / K_A)$ values at 15 min and 50 min post stimulation (Fig. 7e). The $\log(\tau)$ values at 50 min exhibited better linear relation with the corresponding relative maxima than those at 15 min (Fig. 7f). This may be due to the fact that the late response more closely resembles the equilibrium condition than the early response. Fourth, the pEC_{50} values of different agonists showed relatively good correlation with their reported pK_i values, regardless of time points measured (Fig. 7g), but neither their on rate (Fig. 7h) nor off rate (Fig. 7i). Fifth, except for oxotremorine and pilocarpine, the $\log(\tau)$ values of the remaining five agonists at 15 min or 50 min were found to be positively correlated with their reported pK_i values (Fig. 7j) or $\log(\text{on rate})$ (Fig. 7k), but negatively with their $\log(\text{off rate})$ (Fig. 7l). Furthermore, the relative maxima of these agonists did not correlate with their pK_i values (Fig. 7m) or $\log(\text{on rate})$ (Fig. 7n), but were negatively correlated with their $\log(\text{off rate})$ (Fig. 7o). Similarly, for other cell lines the relative maxima of these muscarinic receptor agonists also gave rise to a negative linear relation with their off rate (Fig. 8). Collectively, these analyses suggest that

the off rates of muscarinic receptor agonists have a direct impact on their label-free cell phenotypic efficacy.

4. Discussion

To study the biased agonism and label-free cell phenotypic efficacy of muscarinic receptor agonists acting at the endogenous M_3 receptor, we first identified six out of the total 25 different cell lines that gave rise to the comparatively highest mRNA level for the M_3 receptor subtype over other muscarinic receptor subtypes. Given that the relationship between steady state mRNA levels and receptor protein expression at the cell surface plasma membrane might differ not only across the five muscarinic receptor subtypes but also across the different cell lines, the quantitative RT-PCR data may not be sufficient to identify the functional receptor subtype(s) in these cell lines. Thus, we further determined using multiple DMR assays, together with literature mining, that the six cell lines predominantly express functional M_3 receptors.

The endogenous M_3 receptors in the six different cell lines are further used as model systems to examine the label-free whole cell efficacy of a panel of seven muscarinic receptor agonists. DMR agonist assays showed that different agonists gave rise to cell system-dependent potency, efficacy and DMR characteristics. This is largely in line with the fact that the DMR arising from a receptor–ligand interaction is a holistic representation of the signaling pathways being activated (Fang, 2010; Fang, Li, & Peng, 2005; Tran & Fang, 2008) and different cells have distinct cellular signaling circuits (Kenakin, 2012). Interestingly, the label-free pharmacology of muscarinic antagonists is also sensitive to cell background, as evidenced by atropine that was about 20 times more potent at inhibiting the acetylcholine response in HeLa cells than in HCT-15 cells, and by the relative potency of tiotropium over ipratropium in which tiotropium was 147 fold more potent than ipratropium in CCRF-CEM cells, but only 5.5 fold in PC3 cells. Although exact mechanism(s) are unknown, several factors including differences in receptor expression level and receptor interacting proteins (Kenakin, 2012), and the different binding kinetics (k_{on} and K_{off}) of antagonists relative to acetylcholine as well as the DMR assay formats (Deng et al., 2012) may contribute to these cell line-dependent differences.

Different muscarinic receptor agonists are found to display biased agonism in a cell line dependent manner. First, different agonists exhibited distinct time-dependent potency changes in different cell lines. GPCR signaling is known to consist of a series of spatial and temporal events and pathways, each with its own characteristics such as kinetics, dynamics, amplitude and location (Fang, Frutos, & Verklereen, 2008; Kholodenko, 2006; Lohse et al., 2008). Many of these events and pathways not only can contribute to the DMR arising from the receptor activation, but also can be encoded in different time domains of the DMR (Fang, 2011; Tran & Fang, 2008). Furthermore, the DMR signals of the muscarinic receptor agonists, when being active in a specific cell line, remain above the baseline throughout the assay (Fig. 3). Given that the DMR is a readout of cell signaling events under non-equilibrium condition (Deng et al., 2012; Goral, Jin et al., 2011; Goral, Wu et al., 2011), and different DMR events/pathways have different kinetics (Fang et al., 2007; Tran & Fang, 2008), these results suggest that different agonists may have distinct biased agonism. Of note, we cannot rule out the possibility that the binding kinetics, in particular on-rates, may also contribute to this. Second, pathway deconvolution studies showed that the activation of M_3 receptor can mediate signaling beside the G_q pathway. Specifically, the M_3 receptors in HCT-15 cells can mediate signaling through both G_{aq} and G_{as} pathways, while the receptors in HT-29 mediate signaling primarily through the G_{aq} pathway, and also to a small degree a non- G_{as} , G_{aq} , G_{ai} pathway (possibly $G_{12/13}$ pathway). The M_3 receptor is known to primarily mediate signaling through $G_{q/11}$, but also to couple to other G proteins

including G_s , G_i and $G_{12/13}$ (Felder, 1995; Jakubik et al., 2011; Nahorski, Tobin, & Willars, 1997). Third, pathway deconvolution studies also showed that muscarinic agonists display biased agonism in a cellular context dependent manner. For instance, in HCT-15 cells oxotremorine and pilocarpine are two partial agonists for the QIC-sensitive G_q pathway but inactive in the CTx-sensitive G_s pathway, whereas the other agonists appear active in both pathways. Furthermore, oxotremorine M, carbachol, acetylcholine and methacholine resulted in a DMR that reaches the maximal possible in the native HCT-15 cells, but triggered DMR with different amplitudes in the QIC-treated HCT-15 cells. Fourth, our operational model analysis also pointed out that there are different biases at activating the early versus late responses in HCT-15 and HT-29 cells for different agonists. Of note, given that the DMR signal is a holistic response reflecting the activation of a large number of, as yet often undefined, signaling pathways (Fang, 2011, 2013), DMR is viewed to not be the best readout for studies of agonist signaling bias. Canonically for studying pathway-specific signaling one would have isolated independent readouts of the separate pathways, and the biased agonism is inferred from the reversals of rank order of potency or efficacy among agonists for different signaling pathways. Here, we present evidence suggesting that the endogenous M_3 receptors are capable of mediating signaling via multiple pathways, the exact of which is dependent on both agonist and cell line. These results suggest that combining pathway deconvolution with DMR profiling could assist in elucidating the biased agonism of GPCR ligands.

Lastly, we applied the macro-kinetic parameters of muscarinic receptor agonists binding to the M_3 receptors to estimate their biochemical mechanism of action. Correlation analysis between the label-free efficacy obtained and the reported macro-kinetic parameters suggests that the whole cell efficacy of muscarinic receptor agonists, as defined by the relative maxima of DMR responses, was mostly correlated with their macro-off rate. These findings are in line with recent increasing data suggesting that the kinetics, in particular, drug residence time, of drug–target interactions plays an important role in determining efficacy, safety, duration of action, tolerability, indication and therapeutic differentiation (Copeland, Pompliano, & Meek, 2006; Guo, Mulder-Krieger, Ijzerman, & Heitman, 2012; Lu & Tonge, 2010; Singh, Petter, Baillie, & Whitty, 2011; Swinney, 2011; Sykes et al., 2009; Zhang & Monsma, 2009). The direct impact of both on and off rates of GPCR agonists on their whole cell efficacy is also sensible. It is known that GPCRs can display rich behavior, ranging from rapid onset response to transit response (Fang et al., 2008), as well as delayed and persistent responses via the internalized receptors (Calebiro, Nikolaev, Persani, & Lohse, 2010). The off-rates of agonists, in particular those that cannot get into and accumulate inside the cytoplasm of cells through a mechanism (e.g., transporter) other than the endocytosis process, are expected to have more direct impact on receptor behaviors once being internalized. As a matter of fact, the recent agonist removal studies using the perfusion approach showed that certain signaling can propagate even after agonist removal, but some require the agonist occupancy (Goral, Jin et al., 2011); and the whole cell responses of long-acting β_2 -adrenergic receptor agonists, but not the short-acting ones, are insensitive to the agonist removal, suggesting that the agonist residence time is important for its signaling (Calebiro et al., 2010; Goral, Jin et al., 2011). Nonetheless, the present study provides extra evidence supporting the binding kinetics, particularly the drug residence time, as a plausible molecular mechanism defining the whole cell phenotypic efficacy of GPCR ligands. Of note, the impact of ligand binding kinetics on the in vitro whole cell efficacy observed may not be translated directly into the importance in their in vivo efficacy where circulation and metabolism can be extensive (Fang, 2013).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

DMR	dynamic mass redistribution
GPCR	G protein-coupled receptor
HBSS	Hanks' balanced salt solution
RT-PCR	real time polymerase chain reaction
RWG	resonant waveguide grating

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Fig. 1.
Chemical structures of seven muscarinic receptor agonists examined.

Fig. 2.

Characterization of functional muscarinic receptor subtypes in the six native cell lines. (a) The DMR signals of 16 μ M acetylcholine in these cell lines; (b) The DMR dose responses of McN-A-343 in HCT-15; (c) The dose-dependent effect of gallamine on the DMR of 4 μ M acetylcholine in SF268 (the maximal amplitudes within 10 min, “early P-DMR”, or the amplitudes at 50 min post stimulation) or HCT-15 cells (the amplitudes at 15 min or 50 min post stimulation); (d) The dose-dependent inhibition of three antagonists on the DMR amplitudes at 50 min post stimulation with 4 μ M acetylcholine in HCT-15 cells. Data represents mean $\pm$ s.d (n = 16 for a, n = 4 for b to d).

Fig. 3.

The real-time DMR of muscarinic receptor agonists in the six different cell lines. The agonist doses, each at its $1 \times EC_{100}$, were 16 μ M, 32 μ M, 64 μ M, 32 μ M, 64 μ M, 8 μ M, and 8 μ M in HCT-15 cells (a); 16 μ M, 32 μ M, 64 μ M, 16 μ M, 64 μ M, 8 μ M, and 64 μ M in HT-29 cells (b); 16 μ M, 8 μ M, 32 μ M, 32 μ M, 32 μ M, 32 μ M, and 32 μ M in HeLa cells (c); 32 μ M, 8 μ M, 32 μ M, 16 μ M, 32 μ M, 8 μ M and 64 μ M in SF268 cells (d); 16 μ M, 16 μ M, 64 μ M, 32 μ M, 64 μ M, 16 μ M and 64 μ M in PC3 cells (e); 16 μ M, 16 μ M, 64 μ M, 32 μ M, 64 μ M, 16 μ M, and 32 μ M in CCRF-CEM cells (f) for oxotremorine M, acetylcholine, carbachol, metacholine, bethanechol, oxotremorine, and pilocarpine, respectively. Data represents mean $\pm$ s.d. ($n = 16$).

Fig. 4. Time-dependent potency of muscarinic receptor agonists in different cell lines. (a, b) HCT-15; (c, d) HT-29; (e, f) CCRF-CEM; (g, h) PC3. Data represents mean $\pm$ s.d. (n = 4).

Fig. 5.

Pathway biased agonism of muscarinic receptor agonists in HCT-15 cells. (a) 16 μ M acetylcholine; (b) 16 μ M oxotremorine M; (c) 64 μ M carbachol; (d) 32 μ M methacholine; (e) 64 μ M bethanechol; (f) 16 μ M oxotremorine; (g) 64 μ M pilocarpine. Data represents mean $\pm$ s.d. ($n = 4$). (h) One-way ANOVA followed by Tukey's multiple comparison test of the maximal DMR of different agonists in the QIC-treated cells.

Fig. 6. Dose-dependent DMR responses at 50 min post stimulation of the seven agonists in the six different cell lines. (a) HCT-15; (b) HT-29; (c) HeLa; (d) SF268; (e) PC3; (f) CCRF-CEM cells. Data represents mean $\pm$ s.d. (n = 4).

Fig. 7. Correlation analysis of muscarinic receptor agonists in HCT-15 cells. (a) The relation between pEC_{50} at 15 min and 50 min poststimulation. (b) The relation between the relative maxima at 15 min and 50 min poststimulation. (c) Schematic drawing showing distinct pathways contributing differently to the early and late DMR, respectively, with a stronger coupling efficiency towards the late response. (d) The relation between the τ values at 15 min and 50 min poststimulation; wherein the τ values were obtained based on the reported K_i values or the data fitting derived K_A values. (e) The relation between the $\log(\tau / K_A)$ values at 15 min and 50 min. (f) The relation between $\log(\tau)$ and relative maxima. (g) The relation between pK_i and pEC_{50} . (h) The relation between $\log(k_{on})$ and pEC_{50} . (i) The relation between $\log(k_{off})$ and pEC_{50} . (j) The relation between pK_i and $\log(\tau)$. (k) The relation between $\log(K_{on})$ and $\log(\tau)$. (l) The relation between $\log(k_{off})$ and $\log(\tau)$. (m) The relation between pK_i and relative maxima. (n) The relation between $\log(k_{on})$ and relative maxima. (o) The relation between $\log(k_{off})$ and relative maxima. Data represents mean $\pm$ s.d. ($n = 4$). The p (probability) value for determining the slope if it is significantly non-zero was calculated using linear regression with Prism. A p -value below 0.05 means that the difference is statistically significant. All pK_i , K_{on} and K_{off} values were obtained from Sykes et al. (2009).

Fig. 8.

Relation between relative maxima and $\log (K_{off})$ values of muscarinic receptor agonists in different cell lines. (a) HT-29; (b) PC3; (c) CCRF-CEM; (d) SF268; (e) HeLa cells. Data represents mean $\pm$ s.d. ($n = 4$). The p (probability) value for determining the slope if it is significantly non-zero was calculated using linear regression with Prism. A p-value below 0.05 means that the difference is statistically significant. All pK_i , K_{on} and K_{off} values were obtained from Sykes et al. (2009).

Table 1

Cycle threshold values of endogenous muscarinic receptors and control genes obtained using quantitative real time PCR of mRNA samples.

Cell line	CHRM1	CHRM2	CHRM3	CHRM4	CHRM5	HPRT1	ACTB	GAPDH
HCT15	30.8	34.3	26.1	30.4	32.1	21.6 ± 0.3 ^a	17.5 ± 0.2	16.1 ± 0.2
HT29	38.1	35.0	26.0	32.7	> 40	22.0 ± 0.3	17.4 ± 0.3	15.9 ± 0.2
CCRF-CEM	36.1	35.0	26.2	31.3	34.3	21.1 ± 0.2	16.3 ± 0.3	15.2 ± 0.2
HeLa	38.4	35.8	28.6	31.5	32.0	20.6 ± 0.2	15.9 ± 0.2	15.4 ± 0.3
PC3	35.0	35.4	23.1	31.7	31.6	22.8 ± 0.2	18.1 ± 0.3	16.2 ± 0.2
SF268	39.2	32.7	28.0	32.1	32.8	20.3 ± 0.4	15.2 ± 0.4	14.3 ± 0.3
A431	> 40	36.9	>40	33.9	34.1	21.4 ± 0.4	17.5 ± 0.3	16.8 ± 0.3
A549	39.7	34.1	34.4	31.3	32.4	20.7 ± 0.2	16.3 ± 0.3	15.1 ± 0.2
DU145	37.4	34.7	36.4	32.9	31.2	21.2 ± 0.2	17.0 ± 0.2	15.0 ± 0.2
HUVEC	> 40	33.9	38.7	31.3	32.5	22.0 ± 0.4	17.0 ± 0.3	16.1 ± 0.3
HepG2	> 40	34.3	37.8	30.7	31.0	21.5 ± 0.1	17.3 ± 0.3	15.6 ± 0.4

^aMean ± s.d. of four replicates.

Table 2

The pIC₅₀ values of ligands to block or desensitize the DMR of 4 μ M acetylcholine in the six cell lines (n = 4).

	HCT-15	HT-29	HeLa	PC-3	CCRF-CEM	SF268
Acetylcholine	6.65 $\pm$ 0.03	6.65 $\pm$ 0.02	6.32 $\pm$ 0.13	6.48 $\pm$ 0.06	6.20 $\pm$ 0.03	6.69 $\pm$ 0.06
Oxotremorine M	6.53 $\pm$ 0.04	6.29 $\pm$ 0.03	6.23 $\pm$ 0.05	6.20 $\pm$ 0.04	6.31 $\pm$ 0.03	6.40 $\pm$ 0.06
Methacholine	6.30 $\pm$ 0.04	6.26 $\pm$ 0.02	6.04 $\pm$ 0.05	5.84 $\pm$ 0.05	6.12 $\pm$ 0.06	6.53 $\pm$ 0.07
Carbachol	5.71 $\pm$ 0.06	5.71 $\pm$ 0.03	5.56 $\pm$ 0.06	5.50 $\pm$ 0.06	5.72 $\pm$ 0.05	5.76 $\pm$ 0.05
Oxotremorine	5.70 $\pm$ 0.14	5.72 $\pm$ 0.08	5.94 $\pm$ 0.19	5.57 $\pm$ 0.12	5.63 $\pm$ 0.07	5.70 $\pm$ 0.20
Pilocarpine	4.56 $\pm$ 0.14	5.11 $\pm$ 0.10	5.27 $\pm$ 0.38	4.56 $\pm$ 0.08	4.50 $\pm$ 0.05	4.79 $\pm$ 0.16
Bethanechol	4.83 $\pm$ 0.13	4.33 $\pm$ 0.13	4.78 $\pm$ 0.15	4.16 $\pm$ 0.10	4.45 $\pm$ 0.05	4.51 $\pm$ 0.08
Atropine	4.74 $\pm$ 0.04	5.84 $\pm$ 0.03	6.09 $\pm$ 0.26	5.72 $\pm$ 0.23	5.46 $\pm$ 0.16	4.97 $\pm$ 0.15
Ipratropium	6.21 $\pm$ 0.04	6.53 $\pm$ 0.06	6.40 $\pm$ 0.10	6.91 $\pm$ 0.32	6.16 $\pm$ 0.16	6.76 $\pm$ 0.12
Tiotropium	7.76 $\pm$ 0.02	8.22 $\pm$ 0.02	8.34 $\pm$ 0.09	7.66 $\pm$ 0.01	8.33 $\pm$ 0.18	8.25 $\pm$ 0.04

Table 3

The τ , K_A , $\log (\tau / K_A)$, and bias calculated based on the model of operational agonism. The bias was calculated by subtracting the $\log (\tau / K_A)$ at 50 min from the $\log (\tau / K_A)$ at early time point (15 min for HCT-15, and 2 min for HT-29).

		HCT15	HT29
τ (early)	Acetylcholine	40.9 $\pm$ 5.6 (0.93)	31.3 $\pm$ 1.1 (1.00)
	Carbachol	47.2 $\pm$ 8.9 (1.02)	21.6 $\pm$ 7.3 (1.14)
	Methacholine	8.6 $\pm$ 1.9 (1.00)	12.5 $\pm$ 4.6 (1.02)
	Oxotremorine M	32.4 $\pm$ 9.3 (1.03)	13.7 $\pm$ 3.1 (1.30)
	Bethanechol	1.0 $\pm$ 0.04 (1.35)	1.4 $\pm$ 0.1 (1.4)
τ (50 min)	Acetylcholine	26.6 $\pm$ 5.0 (0.98)	83.3 $\pm$ 20.6 (0.99)
	Carbachol	32.9 $\pm$ 6.8 (1.00)	48.9 $\pm$ 12.1 (1.03)
	Methacholine	31.2 $\pm$ 7.5 (0.94)	6.1 $\pm$ 0.7 (1.04)
	Oxotremorine M	54.3 $\pm$ 8.1 (0.91)	68.7 $\pm$ 15.2 (1.06)
	Bethanechol	4.3 $\pm$ 0.86 (0.88)	1.0 $\pm$ 0.02 (1.45)
pK_A (early)	Acetylcholine	4.82	5.22
	Carbachol	3.76	4.29
	Methacholine	5.14	5.12
	Oxotremorine M	4.80	5.24
	Bethanechol	4.71	4.68
pK_A (50 min)	Acetylcholine	5.30	4.48
	Carbachol	4.25	3.73
	Methacholine	4.76	5.26
	Oxotremorine M	4.79	4.25
	Bethanechol	4.02	4.77
$\log (\tau / K_A)$ (early)	Acetylcholine	6.43	6.72
	Carbachol	5.43	5.63
	Methacholine	6.08	6.21
	Oxotremorine M	6.31	6.38
	Bethanechol	4.73	4.83
$\log (\tau / K_A)$ (50 min)	Acetylcholine	6.72	6.40
	Carbachol	5.76	5.42
	Methacholine	6.26	6.05
	Oxotremorine M	6.52	6.09
	Bethanechol	4.66	4.78
Bias (early – 50 min)	Acetylcholine	0.00	0.00
	Carbachol	–0.04	–0.11
	Methacholine	0.11	–0.15
	Oxotremorine M	0.08	–0.02
	Bethanechol	0.36	–0.26

The values in parentheses is n, the slope of the dose response curve, which is mostly unity.