Selectivity of Agonists for the Active State of $M_1$ - $M_4$ Muscarinic Receptor Subtypes

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Abbreviations: ACh, acetylcholine; CHO, Chinese hamster ovary; DMEM, Dulbecco’s Modified Eagle Medium; $EC_{50}$, concentration of agonist eliciting half-maximal response; $E_{\text{max}}$, maximal response; KRB, Krebs Ringer bicarbonate; McN-A-343, 4-I-[3-chlorophenyl]carbamoyloxy)-2-butyltrimethylammonium chloride; Oxo-M, oxotremorine-M; RAi, intrinsic relative activity.
ABSTRACT

We measured the intrinsic relative activity (RA_i) of muscarinic agonists to detect possible selectivity for receptor subtypes and signaling pathways. RA_i is a relative measure of an agonist’s microscopic affinity constant for the active state of a GPCR expressed relative to that of a standard agonist. First, we estimated RA_i values for a panel of agonists acting at the M4 muscarinic receptor coupled to three distinct G-protein pathways - G_i inhibition of cAMP accumulation, G_s stimulation of cAMP accumulation, and G_{a15} stimulation of phosphoinositide hydrolysis. Our results show similar RA_i values for each agonist, suggesting that the same active state of the M4 receptor triggers activation of the three G proteins. We also estimated RA_i values for agonists across M_1 – M_4 muscarinic subtypes stably transfected in CHO cells. Our results show selectivity of McN-A-343 for the M_1 and M_4 subtypes and selectivity of pilocarpine for the M_1 and M_3 subtypes. The other agonists tested lacked marked selectivity among M_1 – M_4 receptors. Finally, we estimated RA_i values from published literature on M_1, M_2 and M_3 muscarinic responses and obtained results consistent with our own studies. Our results show that the RA_i estimate is a useful receptor-dependent measure of agonist activity.
INTRODUCTION

Novel agonists for G protein coupled receptors (GPCRs) are often identified in high throughput screens based on receptor coupling to alternative G-proteins that mobilize Ca^{2+} (e.g., G_{a15}) (see Milligan & Kostenis, 2006 for review). In such a screen, the profile of an agonist may differ from how it behaves when the receptor is coupled to its native G protein (e.g., G_{i}). Also, the E_{max} and EC_{50} values for triggering a response may vary, depending on the signaling pathway and response being measured. If the E_{max} values of a group of agonists differ within an assay, it is impossible to compare agonist activity accurately using potency ratios.

To understand how drug-receptor interactions influence the output of a functional assay, it is useful to consider different hierarchical levels of analysis of drug action (Figure 1). On the surface (Figure 1a), the behavior of an agonist in an assay can be characterized by its EC_{50} and E_{max} values, which depend not only on the receptor, but on other elements in the signaling pathway as just described. At a deeper level of analysis (Figure 1b), one can examine the relationship between the agonist concentration and the activation state of a population of receptors. For instance, at a ligand gated ion channel, this activation function represents the whole cell current or ensemble average. At a GPCR, the corresponding function is known as the stimulus (Furchgott, 1966). The maximal stimulus is equivalent to observed intrinsic efficacy (ε), and the concentration of agonist eliciting a half-maximal stimulus is equivalent to the observed dissociation constant (K_{obs}). Observed affinity (1/K_{obs}) and intrinsic efficacy are more invariant than EC_{50} and E_{max}, yet nonetheless, these parameter are dependent on the G protein, the concentration of GTP, and other elements that physically interact with the receptor (Ehlert, 2000). It is possible to deduce the stimulus through the analysis of a downstream response using Furchgott’s method of partial receptor inactivation (Furchgott, 1966). At an even deeper level of analysis (Figure 1c), one can consider the microscopic affinity constants of the agonist for the
ground and active states of the receptor (Colquhoun, 1998). These parameters are the ultimate
determinants of agonist activity in different assays. It is possible to estimate these parameters at
ligand-gated ion channels, in some instances, through single channel analysis (Colquhoun 1998).
At a GPCR, it is impossible to estimate microscopic constants from the concentration-response
curve, but it is possible to calculate a relative estimate of the microscopic affinity constant of an
agonist for the active state of the receptor. Analysis of the results of a recent modeling study
show that the product of observed affinity (1/K\textsubscript{obs}) and intrinsic efficacy (\(\varepsilon\)) of an agonist
expressed relative to that of a standard agonist (\(\varepsilon(1/K\textsubscript{obs}')\)) is also equivalent to the
corresponding ratio of microscopic affinity constants for the active state of the receptor (\(K_b/K_b'\))
(Ehlert 2008). This ratio is termed, intrinsic relative activity (RA\textsubscript{i}):

\[
RA_i = \frac{\varepsilon(1/K\textsubscript{obs})}{\varepsilon'(1/K\textsubscript{obs}')} = \frac{K_b}{K_b'}
\]

In prior work, we showed how to estimate RA\textsubscript{i} from the concentration-response curves of the
two agonists (Griffin et al., 2007). Thus, although observed affinity and efficacy are complex
functions of microscopic constants, their product yields a simple constant proportional to the
microscopic affinity constant of the agonist for the active state of the receptor.

Having a relative measure of the affinity of the agonist for the active state of a GPCR
enables one to address several questions. For example, if different active states are involved in
coupling to different G proteins, then the estimate of the agonist RA\textsubscript{i} value should change
depending upon the signaling pathway. Also, if the agonist exhibits selectivity for different
receptor subtypes, then its RA\textsubscript{i} value should reflect this selectivity. Moreover, since all that is
required for estimation of RA\textsubscript{i} is the agonist concentration-response curve, it should be possible
to address these questions from prior published data. In the present report, we have tested these
postulates in connection with subtypes of the muscarinic receptor. Using a panel of agonists, we
found little difference in agonist activity for triggering responses through the M\textsubscript{4} receptor
coupled to G\textsubscript{i}, G\textsubscript{s} or G\textsubscript{a15}. In investigating agonist activity at muscarinic subtypes using RA\textsubscript{i}
analysis, we confirmed the selectivity of McN-A-343 for $M_1$ and $M_4$ receptors and also identified pilocarpine as an $M_1$ and $M_3$ selective agonist. Analysis of data from the literature also yielded a similar picture. Our results show that the $RA_i$ parameter is a simple and useful estimate for comparing agonist activity across assays.
METHODS

Cell Culture: Chinese hamster ovary (CHO) cells stably expressing the human muscarinic M₁ and M₄ receptors were obtained from Acadia Pharmaceuticals (San Diego, CA). The expression levels of muscarinic receptors in these cells were approximately 0.1 pmol (CHO M₄), 0.2 pmol/mg protein (CHO M₂), 1.2 pmol/mg protein (CHO M₃) and 1.3 pmol/mg protein (CHO M₁). HEK-293T cells stably expressing Gₐ₁₅ were provided by Dr. Olivier Civelli (University of California, Irvine, CA.). CHO M₁ cells were cultured in F-12 media Kaighn’s modification (F-12K). CHO M₄ and Gₐ₁₅ HEK-293T cells were cultured in Dulbecco’s modified Eagle’s medium with high glucose plus L-glutamine (DMEM). All media was supplemented with 10% fetal calf serum, penicillin-streptomycin (100 units/ml) and G418 (0.4 mg/ml), and cells were cultured at 37°C with 5% CO₂. HEK-293T Gₐ₁₅ cells were also supplemented with puromycin (0.625 µg/ml). A plasmid containing the human M₄ receptor (hM₄) was obtained from the cDNA Resource Center (Missouri University of Science and Technology, Rolla, MO). An empty pcDNA3.1 vector was obtained from Invitrogen (Carlsbad, CA). HEK-293T Gₐ₁₅ cells were transfected with 10 µg of the hM₄ vector (HEK Gₐ₁₅ M₄) or the empty plasmid (HEK Gₐ₁₅ null) using Lipofectamine (5:1 lipofectamine : DNA ratio.) for 48 hours prior to experimentation.

cAMP Accumulation: The effects of muscarinic agonists on forskolin-stimulated cAMP accumulation was measured in CHO M₂ and M₄ cells using a modification of the [³H]adenine pre-labeling method as described by in Griffin et al. (2007). Pertussis toxin-treatment was accomplished by first incubating the cells with the toxin for 16 hr prior to the assay.

Phosphoinositide Hydrolysis: Muscarinic agonist-mediated stimulation of phosphoinositide hydrolysis was measured in adherent CHO cells and suspensions of HEK Gₐ₁₅ cells using a
modification of the [3H]inositol pre-labeling method of Berridge et al. (1982) and the extraction method of Kendall and Hill (1990). A detailed description of the method used for cell suspension experiments is described in Griffin et al. (2007). Confluent CHO M₁ cell monolayers cultured in 24 well-plates or 100mm petri dishes were washed in KRB prior to overnight incubation with [3H]inositol (2 µCi per well). On the morning of the experiment the 24 well-plates were washed twice with KRB. After 15 min incubation with KRB (270 µl) containing LiCl (10 mM), agonists (30 µl) were added for a subsequent 30 min incubation at 37°C in 5% CO₂. The reaction was stopped with 5% perchloric acid (200 µl), and the samples placed on ice. [3H]Inositol phosphates were isolated as described previously (Ehlert et al., 1996).

**Analysis of Agonist Concentration Response Curves:** $E_{\text{max}}$, $EC_{50}$ and Hill slope were estimated from agonist concentration-response curves by nonlinear regression analysis using Prism (GraphPad Software, Inc., San Diego, CA) as described previously (Griffin et al., 2007).

**Estimation of RAᵢ:** The RAᵢ of test agonist $B$ is defined as the product of its observed intrinsic efficacy ($\varepsilon$) and reciprocal of its observed dissociation constant ($K_{\text{obs}}$) divided by that of standard agonist $A$ as described above in equation 1. To avoid confusion, we have rewritten equation 1 below with subscripts to observed intrinsic efficacy ($\varepsilon$) and observed affinity ($K$) to denote the parameters of the standard and test agonists:

$$RA_i = \frac{\varepsilon_B (1/K_B)}{\varepsilon_A (1/K_A)} = \frac{\varepsilon_B K_A}{\varepsilon_A K_B}$$

The derivation of the RAᵢ value and its estimation using either a null method or the operational model have been described in detail previously (Griffin et al., 2007), and step-by-step instructions for estimating RAᵢ using Prism or a spreadsheet have also been described (Ehlert...
A brief summary of the essential steps is given below. Since the RA_i value is a relative measure of agonist activity, we always ran the standard agonist carbachol in each experiment.

**Null method:** Pairs of equiactive log agonist concentrations were estimated for the standard (LOGA) and test (LOGB) agonists as described previously (Ehlert 2008). The following equation was fitted to these data using nonlinear regression analysis:

\[
\text{LOGB} = \frac{10^{\text{LOGA} + \text{LOGP} + \text{LOGRA} + \text{LOGKA}}}{10^x \left(1 - 10^{\text{LOGP} + \text{LOGRA}}\right) + 10^{\text{LOGP} + \text{LOGKA}}}
\]

In this equation, LOGRA denotes the logarithm of the RA_i value, LOGKA denotes the logarithm of the observed dissociation constant of the standard agonist, and LOGP denotes the logarithm of the ratio of observed dissociation constants of the test agonist divided by that of the standard agonist (Log K_B/K_A). LOGKA was set to an arbitrarily high constant value of -1, and regression analysis yields the best estimates of LOGRA and LOGP. It is possible to estimate the logarithm of K_B from the estimate of LOGP and LOGKA, even though the latter is set as an arbitrarily high constant:

\[
\log K_B = \text{LOGP} + \text{LOGKA}
\]

**Operation method:** For decreasing agonist concentration-response curves, like agonist-mediated inhibition of cAMP accumulation, the concentration-response curves of the standard agonist (A) and the various test agonists (B) were fitted simultaneously to equations 5 and 6, respectively, by nonlinear regression analysis:

\[
\text{response} = P - \left( \frac{M(10^{\text{LOGA}})^N}{(10^{\text{LOGA}})^N + \left(10^{\text{LOGA} + 10^{\text{LOGB} + \text{LOGKA}}}\right)^N} \right)
\]
\[
\text{response} = P - \left( \frac{M(10^{\text{LOG}B}N)}{(10^{\text{LOG}B}N + \left( \frac{10^{\text{LOG}B} + 10^{\text{LOG}KB}}{10^{\text{LOG}KB + \text{LOG}RA}} \right)^N) } \right)
\]

In these equations, \( P \) denotes cAMP accumulation in the absence of agonist, \( N \) denotes the transducer slope factor in the operational model, \( \text{LOGR} \) denotes the ratio of the \( \tau \) value of \( A \) divided by its observed dissociation constant (\( \tau_A/K_A \)), \( \text{LOGKB} \) denotes the logarithm of the observed dissociation constant of the test agonist (\( K_B \)) and \( \text{LOGRA} \) denotes the logarithm of \( R_{Ai} \), which is also a function of parameters in the operational model (Griffin et al. 2007):

\[
\text{LOGRA} = \log \left( \frac{\tau_B K_A}{\tau_A K_B} \right) = \log \left( \frac{\tau_B / K_B}{\tau_A / K_A} \right)
\]

Global nonlinear regression analysis is done sharing the estimates of \( N, M, P, \) and \( \text{LOGR} \) among the curves, and unique estimates of \( \text{LOGRA} \) and \( \text{LOGKB} \) are obtained for each test agonist. If the standard agonist is a full agonist, the parameter \( \text{LOGKA} \) is set as a constant at an arbitrarily high value during regression analysis (e.g., -1).

For increasing agonist concentration-response curves, like agonist mediated simulation of phosphoinositide hydrolysis, the concentration-response curve of the standard agonist and the various test agonists were fitted simultaneously to equations 8 and 9, respectively, by nonlinear regression analysis:

\[
\text{response} = \frac{M(10^{\text{LOG}A}N)}{(10^{\text{LOG}A}N + \left( \frac{10^{\text{LOG}A} + 10^{\text{LOG}KA}}{10^{\text{LOG}KA + \text{LOG}RA}} \right)^N) }
\]

\[
\text{response} = \frac{M(10^{\text{LOG}B}N)}{(10^{\text{LOG}B}N + \left( \frac{10^{\text{LOG}B} + 10^{\text{LOG}KB}}{10^{\text{LOG}KB + \text{LOG}RA}} \right)^N) }
\]
Global nonlinear regression analysis is done as described above for decreasing concentration-response curves, with the exception that the regression equations lack the parameter $P$.

**Operational method for HEK $G_{\alpha15}$ $M_4$ cells:** As described below, HEK $G_{\alpha15}$ $M_4$ cells express low levels of an endogenous $M_3$ receptor in addition to the transiently transfected $M_4$ receptor, indicating that the muscarinic phosphoinositide response in these cells is caused by activation of both $M_3$ and $M_4$ muscarinic receptors. To estimate the $R_A$ value corresponding to the $M_4$ component, we analyzed the agonist concentration-response curves in HEK $G_{\alpha15}$ $M_4$ and HEK $G_{\alpha15}$ null cells simultaneously according to the following two equations, respectively:

$$
\text{response}_{3+4} = \frac{M}{1 + \frac{N}{S_{3+4}}}
$$

$$
\text{response}_3 = \frac{M}{1 + \frac{N}{S_3}}
$$

in which $S_{3+4}$ denotes a parameter proportional to the combined stimulus elicited by activation of both $M_3$ and $M_4$ receptors in HEK $G_{\alpha15}$ $M_4$ cells:

$$
S_{3+4} = \frac{\tau_3 10^{\text{LOGX}}}{10^{\text{LOGX}} + 10^{\text{LOGK}_3}} + \frac{\tau_4 10^{\text{LOGX}}}{10^{\text{LOGX}} + 10^{\text{LOGK}_4}}
$$

and $S_3$ denotes a parameter proportional to the stimulus elicited by activation of the $M_3$ receptor in HEK $G_{\alpha15}$ null cells:

$$
S_3 = \frac{\tau_3 10^{\text{LOGX}}}{10^{\text{LOGX}} + 10^{\text{LOGK}_3}}
$$

The derivation of equations 10 – 13 is given under “Appendix.” Regression analysis was done sharing the estimates of $N, M, \tau_3$ and $\text{LOGK}_3$ between the curves and obtaining unique estimates of $\tau_4$ and $\text{LOGK}_4$ for the data measured in HEK $G_{\alpha15}$ $M_4$ cells. With regard to full agonists in HEK $G_{\alpha15}$ $M_4$ cells, the estimates of $K_4$ and $\tau_4$ are unreliable, and sometimes it was necessary to
set $K_d$ as a constant at an arbitrarily high value to obtain a fit. Regardless, the ratio of $\tau_d/K_d$ can be estimated accurately. Knowing the ratio of $\tau/K$ for the test agonist and standard agonist for a given response (i.e., $M_3$ or $M_4$), it is possible to estimate the corresponding RA_i values using equation 7 above.

**Estimation of RA_i from published studies:** In most instances (11 out of 19), we calculated RA_i values from published concentration-response curves. To make this calculation, we carefully estimated the response values and agonist concentrations from published figures of agonist concentration-response curves. We then calculated the RA_i values from these estimated concentration-response data using the operational method described above. In the remainder of cases (8 out of 19), only the EC_{50} and E_{max} values of the agonist were available from the literature. In these cases, we used the simple calculation for the estimation of RA_i as described previously (Ehlert et al., 1999; Griffin et al., 2007):

$$RA_i = \frac{E_{max-B}EC_{50-A}}{E_{max-A}EC_{50-B}}$$

in which the subscripts refer to the parameters of the standard ($A$) and test ($B$) agonists. This calculation is completely valid if the Hill slopes of the agonist concentration-response curves are equal to one or if the E_{max} values of the agonists are the same, in which case the RA_i is equivalent to the potency ratio regardless of the Hill slopes. In six of the studies where the simple calculation (i.e., equation 14) was used, the data were from studies on second messenger responses in cell lines transfected with subtypes of the muscarinic receptor. We have found that agonists typically exhibit Hill slopes close to one in these types of experiments, suggesting that the simple calculation was valid in these instances. In the remaining two cases (R-aceclidine in the rabbit vas deferens (Eltze et al., 1993 and McN-A-343 in guinea pig right atrium, Lambrecht et al., 1993), the E_{max} values of the agonists were 86 and 59% of the standard agonist, respectively. We expect the simple calculation of RA_i to be valid in the case of R-aceclidine.
because its $E_{\text{max}}$ is close to 100%. If the Hill slope of McN-A343 differs from that of carbachol in the right atrium substantially, then the simple estimate of RA, could be in error by two to threefold (see Ehlert et al., 1999).

**Drug and Chemicals:** Drugs and chemicals were obtained from the following sources: $[^{3}\text{H}]$adenine and $[^{3}\text{H}]$inositol, PerkinElmer Life and Analytical Sciences (Boston, MA); F-12K, DMEM, trypsin-EDTA and Lipofectamine (Invitrogen, Carlsbad, CA); G418 (Invivogen, San Diego, CA); arecoline, carbachol, McN-A-343, and oxotremorine-M, pilocarpine (Sigma-Aldrich, St. Louis, MO). The enantiomers of aceclidine were synthesized and resolved as described by Ringdahl et al. (1979).
RESULTS

Analysis of agonist activity at the M
muscarinic receptor signaling through different G
proteins

To investigate how the activity of specific agonists may be modified by the G protein through which the M
receptor signals we tested a panel of muscarinic agonists for their ability to elicit responses through M
receptor coupling to G, G, and G
. The panel of compounds included agonists with varying structure, efficacy and potency. The standard compound to which the RA values of the other agonists were normalized was carbachol, selected because of its similar structure to the endogenous neurotransmitter, acetylcholine. Oxotremorine-M (oxo-M) was selected as an example of a highly efficacious muscarinic agonist (Fisher and Bartus, 1985). McN-A-343 was investigated as an example of a subtype-selective agonist. This compound was originally described as a sympathetic ganglionic stimulant (Roszkowski, 1961) and has been more recently shown to exhibit selectivity for M and M receptors (Lazareno and Birsdall, 1993). The enantiomers of aceclidine (Ringdahl et al., 1982) were selected as rigid analogs of acetylcholine. The racemate has been used as a treatment for glaucoma (Fechner et al., 1971). Also tested were the partial agonist, pilocarpine, and arecoline, the natural alkaloid from betel nuts.

M
receptor mediated inhibition of cAMP accumulation: Agonist activity for signaling through the M
receptor coupled to G was tested in CHO M
cells by measuring inhibition of forskolin (10 μM) stimulated cAMP accumulation (Figures 2a and 2b). Carbachol, S-aceclidine and
McN-A-343 all produced concentration-response curves with similar potency and maximal effect. Oxo-M was slightly more potent than carbachol, but shared a similar maximal response, whereas arecoline had a similar potency as carbachol but a slightly decreased $E_{\text{max}}$. R-aceclidine had a response both lower in potency and maximum effect compared to carbachol. Pilocarpine exhibited an $EC_{50}$ at least two log units less potent than carbachol but displayed an increased maximal effect, although pilocarpine was not tested at higher concentrations. It is possible that pilocarpine causes a non-muscarinic receptor mediated inhibition of cAMP accumulation at high concentrations as has been previously seen with other agonists (e.g., R-acetidine in CHO cells (Griffin et al., 2007)). The $E_{\text{max}}$, $EC_{50}$ and Hill slope of each agonist are summarized in Table 1.

**M₄ receptor mediated stimulation of cAMP accumulation:** It has been shown that the cAMP response to muscarinic agonists in CHO M₂ and M₄ cells is biphasic. Low concentrations of agonist mediate inhibition of cAMP accumulation, whereas stimulation of cAMP accumulation occurs at higher concentrations of agonist (Mistry et al., 2005). The more potent inhibition of cAMP accumulation is prevented by pretreatment with pertussis toxin, which unmask the $G_s$ dependent stimulation of cAMP accumulation. The role of $G_s$ has been confirmed in siRNA studies (Michal et al., 2007). We investigated the ability of muscarinic agonists to enhance the cAMP accumulation elicited by a low concentration of forskolin (0.1 µM) in CHO M₄ cells treated with pertussis toxin (see Figures 3a and b and Table 2). Oxo-M stimulated an increase in cAMP accumulation with a maximal effect and potency significantly higher than those of carbachol, whereas the $E_{\text{max}}$ and potency of S-aceclidine were lower than those of carbachol. Both McN-A-343 and R-aceclidine failed to produce substantial, concentration-dependent increases in cAMP accumulation. The potency of carbachol for inhibiting cAMP accumulation is
more than 1.3 log units higher than that for stimulating cAMP accumulation in pertussis toxin-treated cells, illustrating the low sensitivity of the CHO M₄ Gₛ assay. This reduced sensitivity can account for the inability of the partial agonists to trigger a response in this assay, rather than inferring a selectivity based on the agonist-receptor-G protein interaction.

*M₄ receptor-mediated phosphoinositide hydrolysis via Gα₁₅:* Offermans and Simon (2001) have described how Gα₁₅ can couple a wide variety G protein coupled receptors to phospholipase C-β. Consequently, we investigated the ability of muscarinic agonists to stimulate the production of inositol phosphates in HEK Gα₁₅ cells transiently transfected with the M₄ receptor (HEK Gα₁₅ M₄ cells). Figure 4a shows the concentration-response curves of the five agonists tested in the HEK Gα₁₅ M₄ cells. Carbachol and S-aceclidine displayed full agonism with similar potency and Eₘₐₓ. Oxo-M also behaved as a full agonist, but showed increased potency with its concentration-response curve located over one log unit to the left of carbachol. McN-A-343 was as potent as carbachol at stimulating phosphoinositide hydrolysis, but had a decreased Eₘₐₓ, whereas R-aceclidine exhibited both lower potency and Eₘₐₓ. Table 3 lists the Eₘₐₓ, EC₅₀ and Hill slope values of agonists for these responses.

In a prior study, we showed that an endogenous M₃ muscarinic receptor elicits a weak phosphoinositide response in the HEK Gα₁₅ cell (Griffin et al., 2007). Muscarinic responses measured in HEK Gα₁₅ cells transiently transfected with the M₄ receptor, therefore, should represent the sum of M₃ and M₄ responses. To quantify the magnitude of the M₃ component, we measured agonist stimulated phosphoinositide hydrolysis in HEK Gα₁₅ cells transfected with an empty pcDNA3.1 vector (HEK Gα₁₅ null, see Fig. 4b). Generally, the activities of all of the agonists were much less in these cells. Oxo-M produced a maximal response similar to that of
carbachol, but exhibited 10-fold greater potency. S-aceclidine exhibited similar potency to carbachol, but had a lower $E_{\text{max}}$. Neither McN-A-343 nor R-aceclidine produced measurable, concentration-dependent agonism in the HEK $G_{a15}$ null cells. Control experiments with HEK $G_{a15}$ M4 cells treated with pertussis toxin show a lack of contribution of $G_{i/o}$ signaling to the phosphoinositide hydrolysis measured upon stimulation by the muscarinic agonists (data not shown).

**Estimation of agonist $RA_i$ values for M4 responses elicited through $G_i$, $G_s$ and $G_{a15}$:** The $RA_i$ values of agonists for eliciting responses through $G_i$ (Figure 2), $G_s$ (Figure 3) and $G_{a15}$ (Figure 4) were estimated using both the operational and null methods as described under “Materials and Methods”. An additional analysis was done using the operational model for those agonists that elicited a significant response in both the HEK $G_{a15}$ M4 and HEK $G_{a15}$ null cells (carbachol and Oxo-M). The concentration-response curves from both cell lines were analyzed simultaneously sharing the estimates of the M3 parameters between both curves and using only the HEK $G_{a15}$ M4 cells for estimation of the M4 parameters. In this manner, the M4 component of the phosphoinositide response in HEK $G_{a15}$ M4 cells was determined. Further details of the calculations are given under “Materials and Methods.” This analysis enabled us to estimate two $RA_i$ values for an agonist; one for the M3 response and one for the M4 response. This careful analysis ultimately showed that the estimate of $RA_i$ value for the M4 component in the HEK $G_{a15}$ M4 cells was practically the same as that estimated assuming that the entire response was elicited by the M4 receptor. Presumably, the endogenous M3 response was too insensitive to influence the M4 response significantly.
A summary of the RA\textsubscript{i} estimates is shown in Figure 5 and the corresponding RA\textsubscript{i} values are also listed in Tables 1-3. Oxo-M exhibited the highest RA\textsubscript{i} values, whereas carbachol, S-aceclidine and McN-A-343 all exhibited values similar to each other, but somewhat lower than that of oxo-M. R-aceclidine exhibited the lowest RA\textsubscript{i} values. None of the agonists exhibited a marked difference in activity for eliciting M\textsubscript{4} responses through the three different G proteins. No RA\textsubscript{i} value was calculated for McN-A-343 and R-aceclidine in the CHO M\textsubscript{4} G\textsubscript{s} assay because of the immeasurable response to these agonists.

Comparison of agonist activity across M\textsubscript{1} - M\textsubscript{4} muscarinic receptors

We used our RA\textsubscript{i} estimates to compare the activity of agonists across the M\textsubscript{1} – M\textsubscript{4} subtypes of the muscarinic receptor. For this analysis, we used data generated from our lab in which the test and standard agonists were assayed in the same experiment to minimize variation between experiments. Most of the RA\textsubscript{i} estimates for the M\textsubscript{2} receptor were taken from Griffin et al. (2007) in which M\textsubscript{2} receptor mediated inhibition of forskolin-stimulated cAMP accumulation was measured. We ran additional experiments with arecoline and pilocarpine, and the combined results are given in Table 5. The data for the M\textsubscript{3} receptor are from (Ehlert et al., 1999). Additional data on the M\textsubscript{1} receptor was obtained and is described next to give a complete picture of activity across the M\textsubscript{1} – M\textsubscript{4} subtypes.

Our data on agonist mediated stimulation of phosphoinositide hydrolysis in CHO M\textsubscript{1} cells is shown in Figures 6\textsubscript{a} and 6\textsubscript{b}. Most of the agonists exhibited a similar maximal response except for the enantiomers of aceclidine whose E\textsubscript{max} values were moderately lower. Also, most of the agonists exhibited similar potency with the striking exception of oxo-M, which exhibited
approximately 10-fold greater potency than carbachol. The potency of pilocarpine was approximately one fourth that of carbachol. These data are summarized in Table 4.

**Agonist RA\textsubscript{i} values at M\textsubscript{1} - M\textsubscript{4} muscarinic receptors:** A summary of the RA\textsubscript{i} estimates for agonists across M\textsubscript{1} - M\textsubscript{4} muscarinic receptors is shown in Figure 7. All values were estimated using the operational method. For this analysis, RA\textsubscript{i} values were estimated from phosphoinositide assays in CHO M\textsubscript{1} and CHO M\textsubscript{3} cells and from cAMP assays on CHO M\textsubscript{2} and CHO M\textsubscript{4} cells, in which the inhibition of cAMP accumulation elicited by forskolin was measured. Oxo-M displayed increased agonist activity relative to carbachol across the M\textsubscript{1} - M\textsubscript{4} muscarinic subtypes, with an especially high RA\textsubscript{i} value of 30 at the M\textsubscript{1} receptor and values of 4.0 to 6.6 at the other subtypes. S-Aceclidine, arecoline and R-aceclidine exhibited approximately uniform activity at the M\textsubscript{1} – M\textsubscript{4} subtypes. The former two compounds had activity similar to carbachol, whereas R-aceclidine exhibited approximately one-tenth the activity of carbachol. The most selective compounds were McN-A-343 and pilocarpine. McN-A-343 exhibited high selectivity for the M\textsubscript{1} and M\textsubscript{4} subtypes and much lower activity at the M\textsubscript{2} (0.0020) and M\textsubscript{3} (0.019) subtypes. The RA\textsubscript{i} values of McN-A-343 at the M\textsubscript{1} and M\textsubscript{4} subtypes were comparable to those of carbachol. Pilocarpine exhibited activity less than carbachol, but showed selectivity between the muscarinic subtypes; its RA\textsubscript{i} values for the M\textsubscript{1} (0.49) and M\textsubscript{3} (0.15) subtypes were much higher than those for the M\textsubscript{2} (0.015) and M\textsubscript{4} (0.013) receptors. One way analysis of variance showed no significant differences among the log RA\textsubscript{i} values of S-aceclidine (F\textsubscript{3,12} = 1.16; P = 0.37) and R-aceclidine (F\textsubscript{3,12} = 2.40; P = 0.12) across the M\textsubscript{1} – M\textsubscript{4} receptor subtypes. In contrast, Oxo-M (F\textsubscript{3,12} = 43.29; P = 1.03 x 10\textsuperscript{-6}), McN-A-343 (F\textsubscript{3,12} = 105.9; P = 8.6 x 10\textsuperscript{-10}), pilocarpine (F\textsubscript{3,10} = 18.30; P = 2.2 x 10\textsuperscript{-4}) and arecoline (F\textsubscript{3,10} = 4.76 P = 0.0260) exhibited significant
differences in their log $R_{Ai}$ values across receptor subtypes. Post hoc comparisons using T tests with the Bonferonni adjustment showed that Oxo-M exhibited selectivity for $M_1$ receptors relative to $M_2 - M_4$ ($P < 0.001$), McN-A-343 exhibited selectivity for $M_1$ and $M_4$ relative to $M_2$ and $M_3$ ($P < 0.001$), and pilocarpine exhibited selectivity for $M_1$ and $M_3$ relative to $M_2$ and $M_4$ ($P < 0.01$). Post hoc comparisons failed to identify significant differences among the log $R_{Ai}$ values of arecoline at the $M_1 - M_4$ subtypes.

**Estimation of $R_{Ai}$ values from published data:** Since the estimation of $R_{Ai}$ only requires the agonist concentration-response curve, it should be possible to estimate $R_{Ai}$ values from previously published data for a variety of responses and determine how invariant the estimate is for a given agonist at a given receptor subtype. To investigate this issue, we calculated the $R_{Ai}$ values of selected agonists for eliciting responses through $M_1$, $M_2$ and $M_3$ muscarinic receptors. Five published studies were used to compare agonist activity at the $M_1$ receptor in addition to our own just described. Agonist stimulated phosphoinositide hydrolysis was analyzed from studies by Richards and Giersbergen (1995) (CHO M1), Schwarz et al., (1993) (CHO M1) and Mei et al., (1991) (B82 fibroblasts transfected with the $M_1$ receptor), and agonist stimulated GTPase activity in CHO M1 cells (Lazareno and Birdsell, 1993) was also analyzed. We also examined the data of Eltze et al., (1993) on $M_1$ receptor-mediated inhibition of electrically stimulated contraction in rabbit vas deferens. There is some question, however, that this response may be mediated by the $M_4$ receptor as described under “Discussion.” Four studies on cell lines, three on myocardial homogenates, and two on the isolated left atrium were selected for comparison of $M_2$ $R_{Ai}$ values. The studies on cell lines included experiments on the inhibition of cAMP accumulation in CHO M2 cells by Griffin et al. (2007), Mistry et al. (2005), McKinney et al.
(1991) and Wang and El-Fakahany (1993). The studies on inhibition of adenylate cyclase activity in myocardial homogenates included those of Ehlert (1985), Keen and Nahorski (1988) and Ehlert et al. (1996). The studies on the isolated, guinea-pig, left atrium were from Christopoulos and Michelson (1997) and Lambrecht et al., (1993). RAi values for the M3 receptor were estimated from studies measuring contraction in the guinea-pig ileum and phosphoinositide hydrolysis in cells and tissues. The data on phosphoinositide hydrolysis were from Ek & Nahorski (1988, parotid gland and ileum), Matsumoto et al. (1994) (ciliary muscle) and Ehlert et al. (1999) (CHO M3 cells). The data on the contractility of the ileum was from Ringdahl et al. (1982), Hanin et al. (1966) and Ehlert et al. (1999). RAi values were calculated as described under “Methods” and plotted as scatter plots for comparison in Figure 8. We have indicated those values that were calculated from the rabbit vas deferens with an asterisk because this tentative M1 response may actually be an M4 response. If the Emax of the standard and reference agonist were the same, the RAi was estimated as the potency ratio (see Griffin et al., 2007).

The greatest variation in RAi values was noted at the M2 receptor (standard deviation of Log RAi = 0.45; 2.8-fold), the least variation at the M3 receptor (standard deviation of Log RAi = 0.14; 1.4-fold) and intermediate variation at the M1 receptor (standard deviation of Log RAi = 0.39; 2.5-fold). One-way analysis of variance revealed no significant differences in the Log RAi values of oxotremorine (F2,6 = 2.31; P = 0.18), S-aceclidine (F2,5 = 3.77; P = 0.10), R-aceclidine (F2,5 = 1.93; P = 0.24) and arecoline (F2,11 = 1.26; P = 0.32) at the M1 – M3 subtypes. In contrast, Oxo-M (F2,11 = 5.78; P = 0.017), McN-A-343 (F2,10 = 43.69; P = 1.14 x 10^{-5}) and pilocarpine (F2,9 = 9.81; P = 0.005) exhibited significant differences at the M1- M3 subtypes. Oxo-M had geometric mean RAi values of 15.5, 8.6 and 3.0 at the M1, M2 and M3 subtypes, respectively,
suggesting increased activity at the \( M_1 \) and \( M_2 \) receptors relative to \( M_3 \). McN-A-343 exhibited the greatest variation in \( R_{A_i} \) values across subtypes (172-fold) with a geometric mean of 0.70 at the \( M_1 \) receptor, and lower values of 0.0041 and 0.023, at \( M_2 \) and \( M_3 \) receptors, respectively. The corresponding \( R_{A_i} \) values for pilocarpine at the \( M_1 - M_3 \) subtypes are 0.63, 0.012 and 0.19 suggesting selectivity primarily for \( M_1 \) and \( M_3 \) receptors over \( M_2 \).

Assessment of \( R_{A_i} \) values for an agonist within the same receptor type highlights differences between studies. \( R_{A_i} \) values for R-aceclidine at the \( M_1 \) receptor vary from 0.017 in the rabbit vas deferens of Eltze et al., (1993) to 0.22 in CHO \( M_1 \) cell data from this study. The variation in \( R_{A_i} \) estimates at the \( M_1 \) receptor is also seen for oxotremorine, with \( R_{A_i} \) values ranging from 3.2 at the \( M_1 \) receptor in murine fibroblasts by Mei et al., (1991) to 50 calculated from GTPase activity in CHO \( M_1 \) cells by Lazareno et al., (1993). As shown in Figure 8, the \( R_{A_i} \) value (6.1) for pilocarpine from Lazareno et al., (1993) was also much higher than that estimated for pilocarpine in four other studies analyzed (0.49, 0.33, 0.35, 0.28), in which phosphoinositide hydrolysis was measured in either CHO \( M_1 \) or B82 \( M_1 \) cells. Oxo-M, McN-A-343, S-aceclidine and arecoline show less variation in \( R_{A_i} \) values between the \( M_1 \) studies evaluated.

The \( R_{A_i} \) values for oxo-M at the \( M_2 \) receptor vary from 2.7 (Ehlert et al., 1996) to 30 (Ehlert 1985). These studies both investigated cardiac adenylate cyclase activity but in different species (rat and rabbit, respectively). The \( R_{A_i} \) values for McN-A-343, oxo-M, arecoline, R-aceclidine and pilocarpine also show greater than a log unit range across \( M_2 \) studies. S-aceclidine has the least difference in \( R_{A_i} \) estimates of all compounds illustrated in Figure 8, with a standard deviation of log \( R_{A_i} \) values of 0.21. As described above, the variance in agonist \( R_{A_i} \) values is substantially decreased when surveying \( M_3 \) based assays.
DISCUSSION

The RA\textsubscript{i} value is a relative measure of the microscopic affinity constant of an agonist for the active state of the receptor. Therefore, if different active states are involved in the coupling of a GPCR to different G proteins, then different RA\textsubscript{i} values might be expected. A panel of muscarinic agonists, carbachol, oxo-M, McN-A-343, S-aceclidine and R-aceclidine, were assessed for possible selectivity for different active states of the M\textsubscript{4} receptor coupling to G\textsubscript{i}, G\textsubscript{s} or G\textsubscript{a15}. Our data with the M\textsubscript{4} receptor provide no evidence for different active states of the M\textsubscript{4} receptor. This result may suggest that measurement of M\textsubscript{4} activation via G\textsubscript{a15} is an appropriate substitute for estimating agonist activity at the M\textsubscript{4} receptor signaling through G\textsubscript{i}, but it is conceivable that other novel agonists may preferentially direct signaling at the M\textsubscript{4} receptor through one G protein more than another. For example, at the M\textsubscript{2} receptor, it has been shown that McN-A-343 has 10-fold greater activity when activating M\textsubscript{2} receptor signaling via G\textsubscript{a15} versus G\textsubscript{i} (Griffin et al., 2007). Therefore, prior to implementation of a cellular screen based on alternative G protein signaling, it would seem prudent to use RA\textsubscript{i} in conjunction with as many well characterized agonists as possible to evaluate potential differences in signaling caused by alternative G protein coupling. Agonist concentrations required to increase cAMP via M\textsubscript{4} signaling through G\textsubscript{s} were much higher than those required to inhibit forskolin stimulated cAMP via M\textsubscript{4} signaling through G\textsubscript{i}, suggesting a possible physiological irrelevance of the M\textsubscript{4} activation via G\textsubscript{s}. Nonetheless, this pathway does provide an additional example of the use of RA\textsubscript{i} in alternative screening paradigms.

Since the RA\textsubscript{i} value is a relative measure of the microscopic affinity constant of an agonist for the active state of the receptor, its use represents an improvement in prior characterizations of the M\textsubscript{1} – M\textsubscript{4} subtypes requiring the two parameters, EC\textsubscript{50} and E\textsubscript{max}. RA\textsubscript{i} also presents an advantage over the use of potency ratios because RA\textsubscript{i} can be calculated in assays in
which the agonists elicit different maximal responses. A rank order of agonist activity, based on selectivity for the active state, is given in Table 6. Our data on CHO M1 cells generally agrees with published data. Two moderate differences are with regard to arecoline and pilocarpine, which gave a higher level of maximal stimulation (118% and 119% respectively) than previously shown in studies on the phosphoinositide response in CHO M1 cells by Schwarz et al., (1993) (87% and 66% respectively) and Richards and Giersbergen (1995) (85% and 76% respectively).

In this study McN-A-343 displayed increased RAi values at the M1 and M4 receptors compared to M2 and M3. This pattern correlates with previous data indicating selectivity of McN-A-343 for both M1 and M4 muscarinic receptors (Lazareno et al., 1993). Roszkowski (1961) first described the pressor effect of McN-A-343 in cats and suggested that this response was mediated by activation of a neuronal muscarinic receptor (M1) in sympathetic ganglia triggering catecholamine release. In the rabbit vas deferens, McN-A-343 inhibits the contractile response to electrical field stimulation, and this response is blocked potently by the M1 selective antagonist, pirenzepine (Eltze, 1988). It is conceivable that this response is mediated by the M4 receptor because pirenzepine exhibits moderately high affinity for the M4 receptor (pKD = 7.23) in addition to its high affinity for the M1 receptor (pKD = 7.77) (Ehlert et al., 1997). In cell lines, McN-A-343 exhibits greater potency and maximal effect at stimulating GTPase activity in CHO M4 cells compared to that observed in CHO M1 cells, but exhibits much lower activity at the M2 and M3 subtypes (Lazareno et al., 1993).

Pilocarpine exhibited RAi values 0.49, 0.015, 0.21 and 0.01 across M1 – M4 receptors, respectively, indicating selectivity for M1 and M3 receptors compared to M2 and M4. Pilocarpine has previously been shown to exhibit selectivity for the M1 receptor based on its activation of GTPase activity in CHO M1 cells (Lazareno and Birdsall, 1993). More recently Fox et al., (2001) showed pilocarpine’s salivating effect is due to its selective stimulation of M1 and M3 receptors present on salivary glands, and Gautam et al., (2004) described how the salivary effect of
Pilocarpine is prevented in M<sub>1</sub>/M<sub>3</sub> receptor double-knockout mice. These data and those of Hammer et al., (1980) and Buckley and Burnsock (1986) showing high affinity binding sites for pirenzepine in the rat submaxillary gland are consistent with the expression of both M<sub>1</sub> and M<sub>3</sub> receptors in this tissue. Our demonstration of the M<sub>1</sub> and M<sub>3</sub> selectivity of pilocarpine may explain its utility in Sjogren’s syndrome for the treatment of dry mouth. Selectivity of pilocarpine has also been investigated centrally; Bymaster et al., (2003) showed that seizures were induced in mice by pilocarpine activation of the M<sub>1</sub> receptor.

The final section of this report compared RA<sub>i</sub> values for selected agonists in 19 previously published studies dating from Hanin et al., 1966 to Griffin et al., 2007. If two assays are based upon the same receptor, but provide significantly different RA<sub>i</sub> values for a compound it may indicate a difference in the active state of the G protein-receptor complex between the two assays or simply variability. It should be noted that in evaluating historical data there is no knowledge of whether the control agonist was tested within the same experiment as the test agonist, and hence, whether control for possible inter-assay variability was adequate. There is a distinct lack of variation in the estimates of RA<sub>i</sub> by different investigators for agonist activity at the M<sub>3</sub> receptor (Figure 7). With the exception of the RA<sub>i</sub> of arecoline in the ciliary muscle of the rabbit (Matsumoto et al., 1994) all agonists presented very similar RA<sub>i</sub> values across the different studies, which investigated phosphoinositide hydrolysis in cell lines, glands and smooth muscle and contraction in the guinea pig ileum (Hanin et al., 1966; Ringdahl et al., 1979; Ek and Nahorski, 1988; Ehlert et al., 1996; Ehlert et al., 1999). Variability in agonist activity across assays was evident in the M<sub>1</sub> and M<sub>2</sub> sets of data. At the M<sub>1</sub> receptor pilocarpine showed greater than a 20-fold difference between the high value from Lazareno et al., (1993) and the low value from Schwarz et al., (1993). These studies both used CHO M<sub>1</sub> cells, but Lazareno et al., measured GTPase activity, whereas Schwarz and coworkers measured phosphoinositide hydrolysis. The high RA<sub>i</sub> value of pilocarpine in the GTPase assay might be attributed to a
particularly low potency response of carbachol, the standard to which other agonists were compared. RA_i values for all other agonists, except for oxo-M, tested in the study of Lazareno et al., are higher than those calculated from other M_1 based studies, suggesting unusually low activity for carbachol. That pattern of selectivity that we observed in our studies (Figure 6) is generally consistent with data from the literature on the M_1 – M_3 subtypes (Figure 7). That is, both sets of data show that oxotremorine, S-aceclidine, R-aceclidine and arecoline lack selectivity, whereas McN-A-343 exhibits selectivity for M_1 relative to M_2 and M_3, pilocarpine exhibits selectivity for M_1 and M_3 relative to M_2, and oxo-M exhibits selectivity for the M_1 relative to M_3. The data from the literature, however, do not support an M_1 selectivity of oxo-M relative to M_2, perhaps because of variation in RA_i estimates at the M_2 receptor. The RA_i value calculated for R-aceclidine in the M_1 rabbit vas deferens assay (Eltze et al., 1993) is more than ten fold lower than that calculated here in the CHO M_1 cells. R-aceclidine is a good substrate for acetylcholinesterase (Pyttel and Robinson, 1973). Its activity, therefore, may be reduced in the isolated tissue because of cholinesterases, but not in CHO M_1 cells, which lack these enzymes.

The potency and ability of an agonist to turn on a GPCR depends on its microscopic affinity constants for ground and active states of the receptor (Colquhoun, 1998; Kenakin, 2007; Ehlert, 2008). It is currently impossible to determine each microscopic affinity constant from the kinds of data we have analyzed; nonetheless, RA_i does provide a relative estimate of the microscopic affinity constant of the active state of the receptor. This parameter is completely dependent on the properties of the agonist and the receptor and completely independent of G proteins and other elements in the signaling cascade. If there are multiple active conformations of the receptor available to the agonist as well as multiple G proteins or effectors, then the RA_i estimate represents a weighted average, depending on the receptor conformations selected by the ligand and attendant effectors (e.g., G proteins). Although it may seem that the G protein has an influence on the estimate of RA_i, G proteins actually provide a window for detecting different
active conformations of the receptor. With defined experimental systems, it should be possible to estimate RA_i for specific GPCR-G protein pairs, similar to our results shown in Figure 4, making RA_i analysis a powerful tool for quantifying ligand-directed signaling.
REFERENCES


LEGENDS FOR FIGURES

Figure 1: Hierarchical levels of analysis of agonist action: The figure summarizes how agonist activity can be estimated at different, internally consistent levels of analysis. At the most superficial level (a, Level 1), agonist activity is estimated from the $EC_{50}$ and $E_{max}$ values of the measured response. These parameters depend on how the agonist interacts with the receptor as well as various elements in the signaling pathway. The second level of analysis (b) refers to the relationship between the agonist concentration and the proportion of the receptor population in the active state (i.e., stimulus). For a GPCR, this relationship depends on the agonist-receptor interaction as well as the concentration of GTP and proteins that physically interact with the receptor (e.g., G proteins). At the ultimate level of analysis (c, Level 3), activity is governed by the affinity of the agonist for ground and active states of the receptor. The goal of pharmacological analysis is to estimate these purely agonist-receptor dependent parameters from more superficial measurements, like the stimulus and response to an agonist.

Figure 2: Muscarinic agonist mediated inhibition of forskolin (10 µM) stimulated cAMP accumulation in CHO M4 cells: Concentration-response curves for a, carbachol, McN-A-343, S-aceclidine and R-aceclidine and b, carbachol, oxo-M, arecoline and pilocarpine are shown. The data represent the means ± S.E.M. of four to ten experiments, each done in triplicate. The data are expressed relative to the level of stimulation cause by 10 µM forskolin alone.

Figure 3: Muscarinic agonist mediated stimulation of cAMP accumulation in CHO M4 cells treated with pertussis toxin: Concentration-response curves for a, carbachol, oxo-M and S-aceclidine and b, McN-A-343 and R-aceclidine are shown. The data represent the means ±
S.E.M. of four experiments, each performed in triplicate. The data are expressed as a percentage above the level of stimulation caused by 0.1 µM forskolin.

**Figure 4: Muscarinic agonist-mediated phosphoinositide hydrolysis in HEK G\(_{\alpha15}\) cells:** Agonist-mediated phosphoinositide hydrolysis was measured in HEK G\(_{\alpha15}\) M4 cells (a) and HEK G\(_{\alpha15}\) cells (b). The data represent the mean values ± S.E.M. of four experiments, each done in triplicate. The data are expressed relative to the E\(_{\text{max}}\) for carbachol.

**Figure 5: Comparison of the RA\(_i\) values of muscarinic agonists for eliciting different responses through M4 via different G-proteins.** The estimates are from Tables 1, 2 and 3.

**Figure 6. Muscarinic agonist-mediated phosphoinositide hydrolysis in M1 CHO cells:** Agonist-mediated phosphoinositide hydrolysis was measured in CHO cells stably transfected with the human M1 receptor. Concentration-response curves are shown for a, carbachol, McN-A-343, S-aceclidine and R-aceclidine and b, carbachol, oxo-M, arecoline and pilocarpine. Mean values ± S.E.M. of three experiments are shown with each done in triplicate. The data are expressed relative to the E\(_{\text{max}}\) for carbachol.

**Figure 7. Comparison of the RA\(_i\) values of agonists for eliciting responses in CHO cells transfected with M1 - M4 muscarinic receptors:** The estimates are from Tables 1, 4 and 5 and Ehlert et al., (1999).

**Figure 8. Comparison of the RA\(_i\) values of agonists for eliciting different responses in assays for M1, M2 and M3 muscarinic receptors.** RA\(_i\) values of selected agonists were estimated from different experimental assays as described in the text. The estimates were calculated from the published concentration-response curves of Lazareno et al. (1993), Eltze et al.
(1993), Mei et al. (1991), Richard and Giersbergen (1995), Schwarz et al. (1993), Griffin et al. (2007), Ehlert (1985), McKinney et al. (1991), Ehlert et al. (1996), Keen and Nahorski (1998), Christopoulos and Mitchelson (1993), Ehlert et al. (1999), Matsumoto et al. (1994), Ek and Nahorski (1998), Hanin (1966) and Ringdahl et al. (1982), Wang and El-Fakahany (1993) and Lambrecht et al. (1993). Asterisks are used to indicate the RAi values estimated from the study of Eltze et al. (1993) on the rabbit vas deferens because there is a question as to whether this response is M1 as indicated in the figure or M4.
Table 1: Agonist activity for inhibiting forskolin stimulated cAMP accumulation in CHO M₄ cells. The data are from Figures 2a and 2b. The data represent the mean estimates ± SEM. The values in parentheses beneath some of the estimates are the Log mean ± SEM.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>$E_{max}^a$ (%)</th>
<th>$EC_{50}$ (µM)</th>
<th>Hill Slope</th>
<th>RA$_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Null</td>
<td>Operational</td>
</tr>
<tr>
<td>Oxotremorine-M</td>
<td>82 ± 2.4</td>
<td>0.030</td>
<td>0.84 ± .10</td>
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<td></td>
<td>(7.52 ± .07)</td>
<td>(0.76 ± .07)</td>
<td>(0.82 ± .07)</td>
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<tr>
<td>Carbachol</td>
<td>88 ± 2.1</td>
<td>0.23</td>
<td>0.76 ± .06</td>
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<tr>
<td></td>
<td>(-6.63 ± .05)</td>
<td>(0.0)</td>
<td>(0.0)</td>
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<tr>
<td>McN-A-343</td>
<td>80 ± 6.6</td>
<td>0.56</td>
<td>0.65 ± .13</td>
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<tr>
<td></td>
<td>(6.25 ± .17)</td>
<td>(-0.31 ± .12)</td>
<td>(-0.20 ± .12)</td>
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<td>S-Aceclidine</td>
<td>87 ± 5.5</td>
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<td>0.60 ± .09</td>
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<td>(-6.43 ± .14)</td>
<td>(-0.0074 ± .20)</td>
<td>(0.0044 ± .10)</td>
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<td>R-Aceclidine</td>
<td>71 ± 9.1</td>
<td>5.74</td>
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<td></td>
<td>(-5.24 ± .26)</td>
<td>(-1.50 ± .09)</td>
<td>(-1.36 ± .04)</td>
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<td>Arecoline</td>
<td>65 ± 3.7</td>
<td>0.13</td>
<td>1.10 ± .07</td>
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<td>(-6.88 ± .09)</td>
<td>(-0.17 ± .10)</td>
<td>(-0.14 ± .15)</td>
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<tr>
<td>Pilocarpine</td>
<td>83 ± 5.8</td>
<td>9.75</td>
<td>0.88 ± .10</td>
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<td></td>
<td>(-5.01 ± .12)</td>
<td>(-1.89 ± .13)</td>
<td>(-1.87 ± .15)</td>
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</table>

$^a$ Denotes the maximum inhibition of forskolin-stimulated cAMP accumulation.
Table 2: Agonist activity for stimulation of cAMP accumulation in CHO M₄ cells previously treated with pertussis toxin. The data are from Figure 3a. The data represent the mean estimates ± SEM. The values in parentheses beneath some of the estimates are the Log mean ± SEM.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>$E_{\text{max}}^a$ (%)</th>
<th>$EC_{50}$ (µM)</th>
<th>Hill Slope</th>
<th>$RA_i$</th>
</tr>
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<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
<td>Null</td>
</tr>
<tr>
<td>Oxotremorine-M</td>
<td>106 ± 3.8</td>
<td>0.54</td>
<td>1.25 ± 0.21</td>
<td>11.80</td>
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<td>(−6.27 ± 0.06)</td>
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<td>(1.07 ± 0.04)</td>
</tr>
<tr>
<td>Carbachol</td>
<td>78 ± 3.2</td>
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<td>1.26 ± 0.24</td>
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<td></td>
<td>(−5.28 ± 0.07)</td>
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<td>(0.0)</td>
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<tr>
<td>S-Aceclidine</td>
<td>53 ± 5.7</td>
<td>11.8</td>
<td>1 $^b$</td>
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<tr>
<td></td>
<td>(−4.93 ± 0.23)</td>
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<td>(−0.41 ± 0.37)</td>
</tr>
</tbody>
</table>

$^a$ Denotes the maximum stimulation of cAMP accumulation expressed as a percentage of basal cAMP accumulation, which is the amount accumulation in the presence of forskolin (0.1 µM).

$^b$ Hill slope constrained to 1.
Table 3: Agonist activity for stimulating phosphoinositide hydrolysis in HEK G_{alpha}15 M_{4} cells.

The data are from Figure 4. The data represent the mean estimates ± SEM. The values in parentheses beneath some of the estimates are the Log mean ± SEM.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>$E_{\text{max}}^{a}$ (%)</th>
<th>$EC_{50}$ (µM)</th>
<th>Hill Slope</th>
<th>$RA_{i}$</th>
<th>$RA_{i}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Null</td>
<td>Operational $^{b}$</td>
</tr>
<tr>
<td>Oxotremorine-M</td>
<td>97 ± 1.1</td>
<td>0.078</td>
<td>0.82 ± .06</td>
<td>8.01</td>
<td>10.67</td>
</tr>
<tr>
<td></td>
<td>(-7.11 ± .04)</td>
<td></td>
<td></td>
<td>(0.90 ± .04)</td>
<td>(1.028 ± .12)</td>
</tr>
<tr>
<td>Carbachol</td>
<td>97 ± 0.9</td>
<td>0.90</td>
<td>0.82 ± .03</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>(-6.05 ± .03)</td>
<td></td>
<td></td>
<td>(0.0)</td>
<td>(0.0)</td>
</tr>
<tr>
<td>McN-A-343</td>
<td>70 ± 1.4</td>
<td>1.1</td>
<td>0.87 ± .08</td>
<td>0.65</td>
<td>0.44</td>
</tr>
<tr>
<td></td>
<td>(-5.98 ± .06)</td>
<td></td>
<td></td>
<td>(-0.19 ± .07)</td>
<td>(-0.36 ± .05)</td>
</tr>
<tr>
<td>S-Aceclidine</td>
<td>91 ± 1.7</td>
<td>0.62</td>
<td>0.82 ± .07</td>
<td>1.42</td>
<td>1.24</td>
</tr>
<tr>
<td></td>
<td>(-6.21 ± .05)</td>
<td></td>
<td></td>
<td>(0.15 ± .06)</td>
<td>(0.09 ± .06)</td>
</tr>
<tr>
<td>R-Aceclidine</td>
<td>61 ± 1.9</td>
<td>2.7</td>
<td>0.92 ± .13</td>
<td>0.22</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>(-5.56 ± .08)</td>
<td></td>
<td></td>
<td>(-0.65 ± .04)</td>
<td>(-0.87 ± .08)</td>
</tr>
</tbody>
</table>

$^{a}$ Denotes the maximum stimulation of phosphoinositide hydrolysis by carbachol.

$^{b}$ The operational $RA_{i}$ values for oxotremorine-M and carbachol were estimated using equations 10 – 13, whereas those for McN-A-343, S-aceclidine and R-aceclidine were estimated using equations 8 and 9. In each analysis the concentration-response curve of carbachol was analyzed simultaneously as the standard.
Table 4: Agonist activity for stimulating phosphoinositide hydrolysis in CHO M₁ cells. The data are from Figure 6a and 6b. The data represent the mean estimates ± SEM. The values in parentheses beneath some of the estimates are the Log mean ± SEM.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>$E_{\text{max}}$ $^a$ (%)</th>
<th>$EC_{50}$ (µM)</th>
<th>Hill Slope</th>
<th>$RA_{i}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Null</td>
<td>Operational</td>
</tr>
<tr>
<td>Oxotremorine-M</td>
<td>100 ± 2.3</td>
<td>0.041</td>
<td>1.16 ± .13</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(-7.39 ± .05)</td>
<td></td>
<td>(1.51 ± .28)</td>
</tr>
<tr>
<td>Carbachol</td>
<td>98 ± 1.1</td>
<td>1.4</td>
<td>1.29 ± .07</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(-5.86 ± .02)</td>
<td></td>
<td>(0.0)</td>
</tr>
<tr>
<td>McN-A-343</td>
<td>77 ± 1.7</td>
<td>1.9</td>
<td>1.09 ± .09</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(-5.72 ± .04)</td>
<td></td>
<td>(-0.16 ± .06)</td>
</tr>
<tr>
<td>S-Aceclidine</td>
<td>102 ± 1.5</td>
<td>3.8</td>
<td>1.29 ± .07</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(-5.42 ± .03)</td>
<td></td>
<td>(-0.14 ± .11)</td>
</tr>
<tr>
<td>R-Aceclidine</td>
<td>61 ± 0.46</td>
<td>3.1</td>
<td>1.25 ± .03</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(-5.51 ± .01)</td>
<td></td>
<td>(-0.51 ± .11)</td>
</tr>
<tr>
<td>Arecoline</td>
<td>118 ± 2.9</td>
<td>1.7</td>
<td>0.93 ± .08</td>
<td>1.51</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(-5.77 ± .05)</td>
<td></td>
<td>(0.18 ± .04)</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>119 ± 3.1</td>
<td>5.7</td>
<td>0.81 ± .06</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(-5.24 ± .05)</td>
<td></td>
<td>(-0.32 ± .06)</td>
</tr>
</tbody>
</table>

$^a$ Denotes the maximum stimulation of phosphoinositide hydrolysis by carbachol.
Table 5: Agonist activity for inhibiting forskolin stimulated cAMP accumulation in CHO M2 cells. The data represent the mean estimates ± SEM. The values in parentheses beneath some estimates are the Log mean ± SEM. NC denotes not calculated as the E_{max} for the test agonist was the same as the standard carbachol; therefore, RA_{i} values were calculated as the potency ratio.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>E_{max}^{a} (%)</th>
<th>EC_{50} (µM)</th>
<th>Hill Slope</th>
<th>RA_{i}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Null</td>
</tr>
<tr>
<td>Oxotremorine-M</td>
<td>73 ± 2.3</td>
<td>0.047</td>
<td>1.02 ± .06</td>
<td>NC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(-7.32 ± .07)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbachol</td>
<td>73 ± 2.3</td>
<td>0.22</td>
<td>0.90 ± .08</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(-6.65 ± .07)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>McN-A-343</td>
<td>32 ± 5.1</td>
<td>38</td>
<td>1.17 ± .28</td>
<td>0.0022</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(-4.42 ± .13)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-Aceclidine</td>
<td>73 ± 2.3</td>
<td>0.41</td>
<td>0.89 ± .07</td>
<td>NC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(-6.39 ± .10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R-Aceclidine</td>
<td>73 ± 2.3</td>
<td>2.6</td>
<td>0.83 ± .08</td>
<td>NC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(-5.59 ± .08)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arecoline</td>
<td>45 ± 1.9</td>
<td>0.83</td>
<td>1.20 ± .16</td>
<td>0.43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(-6.08 ± .07)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>40 ± 1.4</td>
<td>17</td>
<td>1.11 ± .16</td>
<td>0.014</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(-4.76 ± .06)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^{a} Denotes the maximum inhibition of forskolin-stimulated cAMP accumulation.

^{b} Data previously published in Griffin et al., 2007.
Table 6: Rank order of agonist activity based upon RAᵢ values calculated via the operational method. Data taken from Tables 1, 4 and 5 and Figure 7.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>RAᵢ rank order</th>
</tr>
</thead>
<tbody>
<tr>
<td>M₁</td>
<td>oxo-M &gt; arecoline = carbachol &gt; S-aceclidine = McN-A-343 &gt; pilocarpine &gt; R-aceclidine</td>
</tr>
<tr>
<td>M₂</td>
<td>oxo-M &gt; carbachol &gt; S-aceclidine &gt; arecoline &gt; R-aceclidine &gt; pilocarpine &gt; McN-A-343</td>
</tr>
<tr>
<td>M₃</td>
<td>oxo-M &gt; carbachol = arecoline &gt; S-aceclidine &gt; pilocarpine &gt; R-aceclidine = McN-A-343</td>
</tr>
<tr>
<td>M₄</td>
<td>oxo-M &gt; carbachol = S-aceclidine &gt; arecoline &gt; McN-A-343 &gt; R-aceclidine = pilocarpine</td>
</tr>
</tbody>
</table>
**Level 1:** Agonist concentration-response curve

The agonist concentration-response curve yields estimates of the potency ($EC_{50}$) and maximal effect ($E_{max}$) of the agonist for eliciting a particular response.

**Level 2:** Agonist activation of the receptor population

In a population of receptors, the relationship between receptor activation and the agonist concentration is known as the stimulus. The concentration of the agonist eliciting half-maximal receptor activation is defined as the observed dissociation constant ($K_{obs}$) and the maximal level of receptor activation is known as observed intrinsic efficacy ($\varepsilon$).

**Level 3:** Agonist affinity for ground and active receptor states

The ability of an agonist to bind to and turn on the receptor is determined by its microscopic affinity constants for the ground ($K_a$, $K_q$) and active ($K_b$) states of the receptor. The constant describing the spontaneous formation of the active state is $K_q$. 

Figure 1
Figure 3
Figure 4

**a**

\[ \text{[H]} \text{Inositolphosphates (}\% \text{Carbachol E}_{\text{max}}) \]

Log [Agonist]

- Carbachol
- Oxo-M
- McN-A-343
- S-Aceclidine
- R-Aceclidine

**b**

\[ \text{[H]} \text{Inositolphosphates (}\% \text{Carbachol E}_{\text{max}}) \]

Log [Agonist]

- Carbachol
- Oxo-M
- McN-A-343
- S-Aceclidine
- R-Aceclidine
Figure 7

The graph illustrates the effects of various compounds on $R_A_i$ measured in different conditions labeled $M_1$ to $M_4$. Each compound is represented by a different pattern and color: Carbachol, Oxo-M, McN-A-343, S-Aceclidine, R-Aceclidine, Arecoline, and Pilocarpine. The y-axis represents $R_A_i$ on a logarithmic scale ranging from 0.001 to 100.
Figure 8

- Oxo-M
- Oxotremorine
- McN-A-343
- S-Aceclidine

- R-Aceclidine
- Arecoline
- Pilocarpine
APPENDIX

This appendix describes the derivation of equations 10 – 13 used for the analysis of the concentration-response curves in HEK G_{α15} M_4 and HEK G_{α15} null cells. These equations are based on the operational model (Black and Leff, 1983), which describes the agonist concentration-response curve as a logistic function of the stimulus (s):

\[ \text{response} = \frac{s^N M}{s^N + K_E^N} \]  

15

In this equation, \( N \) denotes the transducer slope factor, \( M \) denotes the maximal response of the system, and \( K_E \) denotes a constant related to the sensitivity of the stimulus-response function. Equation 15 can be rearranged into the following form:

\[ \text{response} = \frac{M}{1 + \left(\frac{s}{K_E}\right)^N} \]  

16

Substituting in a parameter (\( S \)) for \( s/K_E \) yields equations 10 and 11 under “Materials and Methods.” The stimulus is defined according to Furchgott (1966):

\[ s = \frac{X \varepsilon R_T}{X + K} \]  

17

In this equation, \( X \) denotes the concentration of agonist, \( \varepsilon \) denotes the observed intrinsic efficacy of the agonist-receptor complex, \( R_T \) denotes the total receptor concentration and \( K \) denotes the observed dissociation constant of the agonist-receptor complex. Dividing both sides of equation 17 by \( K_E \) yields:

\[ S = \frac{X \tau}{X + K} \]  

18

in which:

\[ \tau = \frac{\varepsilon R_T}{K_E} \]  

19
Equations 18 and 19 provide the basis for equations 12 and 13 under “Materials and Methods.”