0022-3565/09/3281-331–342$20.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
JPET 328:331–342, 2009

Vol. 328, No. 1
145219/3414203
Printed in U.S.A.

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

Katherine W. Figueroa, Michael T. Griffin, and Frederick J. Ehlert

Department of Pharmacology, School of Medicine, University of California, Irvine, Irvine, California (K.W.F., F.J.E.); and Department of Physical Sciences, Chapman University, Orange, California (M.T.G.)

Received August 22, 2008; accepted September 26, 2008

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 the microscopic affinity constant of an agonist 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 M_4 muscarinic receptor coupled to three distinct G-protein pathways: G_i inhibition of cAMP accumulation, G_s stimulation of cAMP accumulation, and $G\alpha_{15}$ stimulation of phosphoinositide hydrolysis. Our results show similar RA_i values for each agonist, suggesting that the same active state of the M_4 receptor triggers the activation of the three G proteins. We also estimated RA_i values for agonists across M_1 to M_4 muscarinic subtypes stably transfected in Chinese hamster ovary cells. Our results show selectivity of McN-A-343 [4-*I*-[3-chlorophenyl]carbamoyloxy)-2-butynyltrimethylammnonium chloride] 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 to 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.

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., $G\alpha_{15}$) (for review, see Milligan and Kostenis, 2006). 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). Furthermore, 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 (Fig. 1). On the surface (Fig. 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 described. At a deeper level of analysis (Fig. 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 (Fig. 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).

This work was supported by National Institutes of Health [Grant GM 69829].
Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
doi:10.1124/jpet.108.145219.

ABBREVIATIONS: GPCR, G protein-coupled receptor; RA_i, intrinsic relative activity; CHO, Chinese hamster ovary; KRB, Krebs Ringer bicarbonate; HEK, human embryonic kidney; McN-A-343, 4-*I*-[3-chlorophenyl]carbamoyloxy)-2-butynyltrimethylammnonium chloride; oxo-M, oxotremorine-M; F-12K, F-12 medium Kaighn's modification.

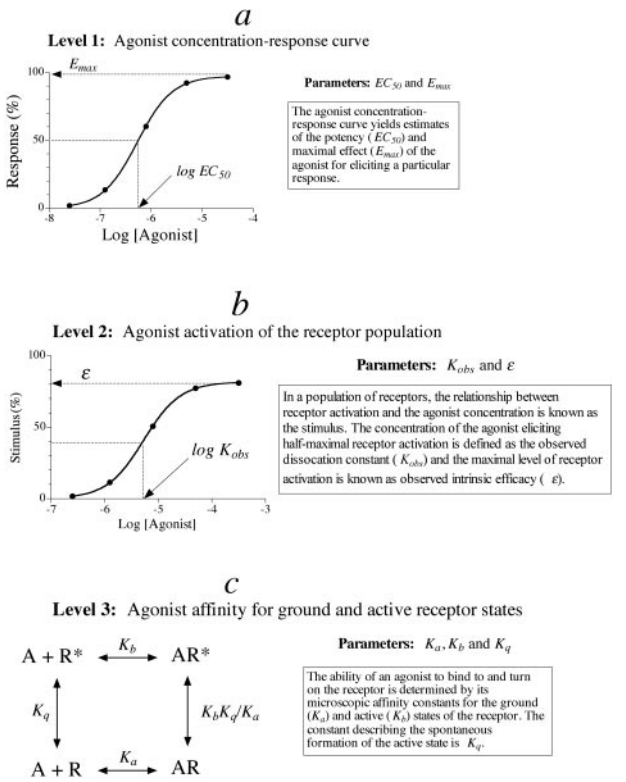

Fig. 1. Hierarchical levels of analysis of agonist action. The figure summarizes how agonist activity can be estimated at different, internally consistent levels of analysis. At the most superficial level (a, Level 1), agonist activity is estimated from the EC_{50} and E_{max} values of the measured 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, such as the stimulus and response to an agonist.

At a GPCR, it is impossible to estimate microscopic constants from the concentration-response curve; however, 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 shows that the product of observed affinity ($1/K_{obs}$) and intrinsic efficacy (ε) of an agonist expressed relative to that of a standard agonist [$\varepsilon'(1/K'_{obs})$] 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_i).

$$RA_i = \frac{\varepsilon(1/K_{obs})}{\varepsilon'(1/K'_{obs})} = \frac{K_b}{K'_b} \quad (1)$$

In prior work, we showed how to estimate RA_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, the estimate of the agonist RA_i value should change depending upon the signaling pathway. In addition, if the agonist exhibits selectivity for different receptor subtypes, its RA_i value should reflect this selectivity. Moreover, because all that is required for estimation of RA_i is the agonist concentration-response curve, it should be possible to address these questions from previously published data. In the present report, we have tested these postulates in connection with the subtypes of the muscarinic receptor. Using a panel of agonists, we found little difference in agonist activity for triggering responses through the M_4 receptor coupled to G_i, G_s, or $G\alpha_{15}$. Upon investigating agonist activity at muscarinic subtypes using RA_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.

Materials and Methods

Cell Culture. Chinese hamster ovary (CHO) cells stably expressing the human muscarinic M_1 and M_4 receptors were obtained from Acadia Pharmaceuticals (San Diego, CA). The expression levels of muscarinic receptors in these cells were approximately 0.1 pmol (CHO M_4), 0.2 pmol/mg protein (CHO M_2), 1.2 pmol/mg protein (CHO M_3), and 1.3 pmol/mg protein (CHO M_1). HEK-293T cells stably expressing $G\alpha_{15}$ were provided by Dr. Olivier Civelli (University of California, Irvine, CA). CHO M_1 cells were cultured in F-12K. CHO M_4 and $G\alpha_{15}$ HEK-293T cells were cultured in Dulbecco's modified Eagle's medium with high glucose plus L-glutamine. All media were 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% CO_2. HEK-293T $G\alpha_{15}$ cells were also supplemented with puromycin (0.625 μg/ml). A plasmid containing the human M_4 receptor 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 $G\alpha_{15}$ cells were transfected with 10 μg of the human M_4 vector HEK $G\alpha_{15}$ M_4 or the empty plasmid HEK $G\alpha_{15}$ null using Lipofectamine (5:1 Lipofectamine/DNA ratio) for 48 h before experimentation.

cAMP Accumulation. The effects of muscarinic agonists on forskolin-stimulated cAMP accumulation were measured in CHO M_2 and M_4 cells using a modification of the [^{3}H]adenine-prelabeling method as described by Griffin et al. (2007). Pertussis toxin treatment was accomplished by first incubating the cells with the toxin for 16 h before the assay.

Phosphoinositide Hydrolysis. Muscarinic agonist-mediated stimulation of phosphoinositide hydrolysis was measured in adherent CHO cells and suspensions of HEK $G\alpha_{15}$ cells using a modification of the [^{3}H]inositol-prelabeling 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 M_1 cell monolayers cultured in 24-well plates or 100-mm Petri dishes were washed in KRB before overnight incubation with [^{3}H]inositol (2 μCi/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% CO_2. The reaction was stopped with 5% perchloric acid (200 μl), and the samples were placed on ice.

[^{3}H]Inositol phosphates were isolated as described previously (Ehlert et al., 1996).

Analysis of Agonist Concentration Response Curves. E_{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 RA_i. The RA_i of test agonist B is defined as the product of its observed intrinsic efficacy (ε) and reciprocal of its K_{obs} divided by that of standard agonist A as described above in eq. 1. To avoid confusion, we have rewritten eq. 1 below with subscripts to observed intrinsic efficacy (ε) and observed affinity (K) to denote the parameters of the standard and test agonists.

$$RA_i = \frac{\varepsilon_B(1/K_B)}{\varepsilon_A(1/K_A)} = \frac{\varepsilon_B K_A}{\varepsilon_A K_B} \quad (2)$$

The derivation of the RA_i 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 RA_i using Prism or a spreadsheet have also been described previously (Ehlert, 2008). A brief summary of the essential steps is given below. Because 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.

$$LOGB = \frac{10^{LOGA+LOGP+LOGRA+LOGKA}}{10^{LOGA}(1 - 10^{LOGP+LOGRA}) + 10^{LOGP+LOGKA}} \quad (3)$$

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 yielded 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.

$$\text{Log}K_B = LOGP + LOGKA \quad (4)$$

Operational 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 eqs. 5 and 6, respectively, by nonlinear regression analysis.

$$\text{Response} = P - \left(\frac{M(10^{LOGA})^N}{(10^{LOGA})^N + \left(\frac{10^{LOGA} + 10^{LOGKA}}{10^{(LOGKA+LOGR)}} \right)^N} \right) \quad (5)$$

$$\text{Response} = P - \left(\frac{M(10^{LOGB})^N}{(10^{LOGB})^N + \left(\frac{10^{LOGB} + 10^{LOGKB}}{10^{(LOGKB+LOGR+LOGRA)}} \right)^N} \right) \quad (6)$$

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

$$LOGRA = \text{Log}\left(\frac{\tau_B K_A}{\tau_A K_B}\right) = \text{Log}\left(\frac{\tau_B/K_B}{\tau_A/K_A}\right) \quad (7)$$

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

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

$$\text{Response} = \frac{M(10^{LOGA})^N}{(10^{LOGA})^N + \left(\frac{10^{LOGA} + 10^{LOGKA}}{10^{(LOGKA+LOGR)}} \right)^N} \quad (8)$$

$$\text{Response} = \frac{M(10^{LOGB})^N}{(10^{LOGB})^N + \frac{(10^{LOGB} + 10^{LOGKB})^N}{(10^{(LOGKB+LOGR+LOGRA)})^N}} \quad (9)$$

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 $G\alpha_{15}$ M_4 Cells. As described below, HEK $G\alpha_{15}$ 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 RA_i value corresponding to the M_4 component, we analyzed the agonist concentration-response curves in HEK $G\alpha_{15}$ M_4 and HEK $G\alpha_{15}$ null cells simultaneously according to the following two equations, respectively,

$$\text{Response}_{3+4} = \frac{M}{1 + \frac{1}{S_{3+4}^N}} \quad (10)$$

$$\text{Response}_3 = \frac{M}{1 + \frac{1}{S_3^N}} \quad (11)$$

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 $G\alpha_{15}$ M_4 cells,

$$S_{3+4} = \frac{\tau_3 10^{LOGX}}{10^{LOGX} + 10^{LOGK3}} + \frac{\tau_4 10^{LOGX}}{10^{LOGX} + 10^{LOGK4}} \quad (12)$$

and S_3 denotes a parameter proportional to the stimulus elicited by activation of the M_3 receptor in HEK $G\alpha_{15}$ null cells.

$$S_3 = \frac{\tau_3 10^{LOGX}}{10^{LOGX} + 10^{LOGK3}} \quad (13)$$

The derivation of eqs. 10 to 13 is given under *Appendix*. Regression analysis was done sharing the estimates of N, M, τ_3, and LOGK3 between the curves and obtaining unique estimates of τ_4 and LOGK4 for the data measured in HEK $G\alpha_{15}$ M_4 cells. With regard to full agonists in HEK $G\alpha_{15}$ M_4 cells, the estimates of K_4 and τ_4 are unreliable. Sometimes, it was necessary to set K_4 as a constant at an arbitrarily high value to obtain a fit. Regardless, the ratio of τ_4/K_4 can be estimated accurately. Knowing the ratio of τ/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 eq. 7 above.

Estimation of RA_i from Published Studies. In most instances (11 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 fig-

ures 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 the cases (8 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),

$$RA_i = \frac{E_{max-B}EC_{50-A}}{E_{max-A}EC_{50-B}} \quad (14)$$

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., eq. 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_{max} is close to 100%. If the Hill slope of McN-A-343 differs from that of carbachol in the right atrium substantially, the simple estimate of RA_i could be in error by 2- to 3-fold (see Ehlert et al., 1999).

Drug and Chemicals. Drugs and chemicals were obtained from the following sources: [^{3}H]adenine and [^{3}H]inositol (PerkinElmer Life and Analytical Sciences, Waltham, MA); F-12K, Dulbecco's modified Eagle's medium, trypsin-EDTA, and Lipofectamine (Invitrogen, Carlsbad, CA); G418 (Invivogen, San Diego, CA); arecoline, carbachol, McN-A-343, and oxotremorine-M (oxo-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_4 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_4 receptor signals, we tested a panel of muscarinic agonists for their ability to elicit responses through M_4 receptor coupling to G_i, G_s, and $G\alpha_{15}$. The panel of compounds included agonists with varying structure, efficacy, and potency. The standard compound to which the RA_i values of the other agonists were normalized was carbachol, selected because of its similar structure to the endogenous neurotransmitter, acetylcholine. 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 shown more recently to exhibit selectivity for M_1 and M_4 receptors (Lazareno and Birdsall, 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., 1975). The partial agonist, pilocarpine, and arecoline, the natural alkaloid from betel nuts, also were tested.

M_4 Receptor-Mediated Inhibition of cAMP Accumulation. Agonist activity for signaling through the M_4 receptor coupled to G_i was tested in CHO M_4 cells by measuring inhibition of forskolin (10 μM)-stimulated cAMP accumulation (Fig. 2, a and b). 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_{max}. *R*-Aceclidine had a response both lower in potency and maximal effect compared with 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 nonmuscarinic receptor-mediated inhibition of cAMP accumulation at high concentrations as has been previously seen with other agonists [e.g., *R*-acelidine in CHO cells (Griffin et al., 2007)]. The E_{max}, EC_{50}, and Hill slope of each agonist are summarized in Table 1.

M_4 Receptor-Mediated Stimulation of cAMP Accumulation. It has been shown that the cAMP response to muscarinic agonists in CHO M_2 and M_4 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 unmasks the G_s-dependent stimulation of cAMP accumulation. The role of G_s

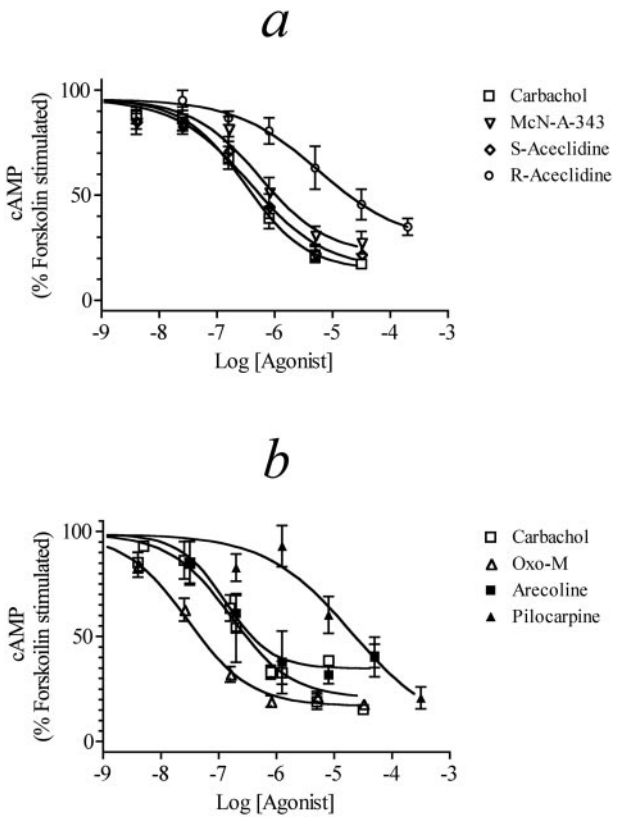

Fig. 2. Muscarinic agonist-mediated inhibition of forskolin (10 μM) stimulated cAMP accumulation in CHO M_4 cells. Concentration-response curves for carbachol, McN-A-343, *S*-aceclidine, and *R*-aceclidine (a) and carbachol, oxo-M, arecoline, and pilocarpine (b) are shown. The data represent the means ± S.E.M. of 4 to 10 experiments, each done in triplicate. The data are expressed relative to the level of stimulation caused by 10 μM forskolin alone.

TABLE 1
Agonist activity for inhibiting forskolin stimulated cAMP accumulation in CHO M_4 cells
The data are from Fig. 2, a and b. The data represent the mean estimates ± S.E.M. The values in parentheses to the right of some of the estimates are the Log mean ± S.E.M.

Agonist	E_{max}[a]	EC_{50}	Hill Slope	RA_i	
				Null	Operational
	%	μM			
Oxotremorine-M	82 ± 2.4	0.030 (−7.52 ± 0.07)	0.84 ± 0.10	5.79 (0.76 ± 0.07)	6.61 (0.82 ± 0.07)
Carbachol	88 ± 2.1	0.23 (−6.63 ± 0.05)	0.76 ± 0.06	1.0 (0.0)	1.0 (0.0)
McN-A-343	80 ± 6.6	0.56 (−6.25 ± 0.17)	0.65 ± 0.13	0.49 (−0.31 ± 0.12)	0.63 (−0.20 ± 0.12)
S-Aceclidine	87 ± 5.5	0.37 (−6.43 ± 0.14)	0.60 ± 0.09	0.98 (−0.0074 ± 0.20)	1.00 (0.0044 ± 0.10)
R-Aceclidine	71 ± 9.1	5.74 (−5.24 ± 0.26)	0.61 ± 0.15	0.071 (−1.50 ± 0.09)	0.040 (−1.36 ± 0.04)
Arecoline	65 ± 3.7	0.13 (−6.88 ± 0.09)	1.10 ± 0.07	0.68 (−0.17 ± 0.10)	0.73 (−0.14 ± 0.15)
Pilocarpine	83 ± 5.8	9.75 (−5.01 ± 0.12)	0.88 ± 0.10	0.013 (−1.89 ± 0.13)	0.013 (−1.87 ± 0.15)

[a] Denotes the maximal inhibition of forskolin-stimulated cAMP accumulation.

has been confirmed in small interference RNA 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 M_4 cells treated with pertussis toxin (see Figs. 3, a 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_{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 M_4 G_s 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.

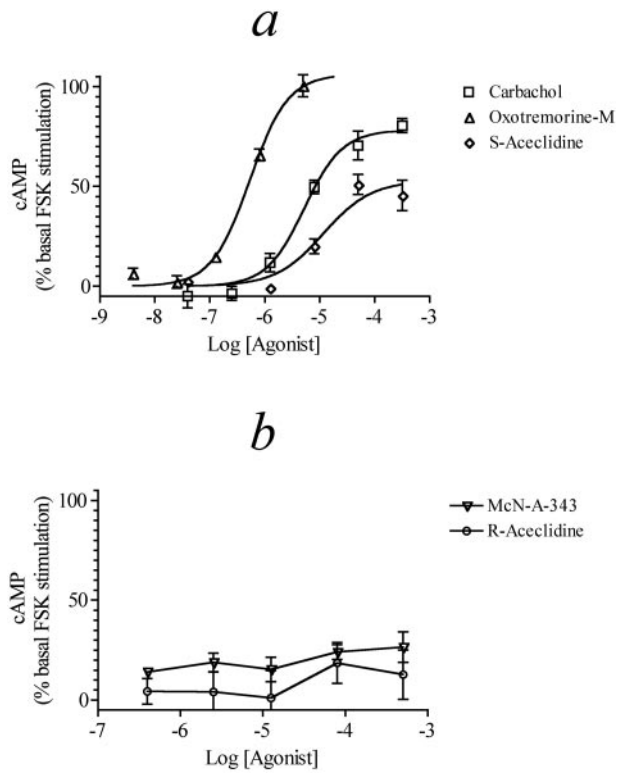

Fig. 3. Muscarinic agonist-mediated stimulation of cAMP accumulation in CHO M_4 cells treated with pertussis toxin. Concentration-response curves for carbachol, oxo-M, and *S*-aceclidine (a) and McN-A-343 and *R*-aceclidine (b) are shown. The data represent the means ± S.E.M. of four experiments, each performed in triplicate. The data are expressed as a percentage above the level of stimulation caused by 0.1 μM forskolin.

M_4 Receptor-Mediated Phosphoinositide Hydrolysis via $G\alpha_{15}$. Offermanns et al. (2001) have described how $G\alpha_{15}$ can couple a wide variety G protein-coupled receptors to phospholipase C-ß. As a consequence, we investigated the ability of muscarinic agonists to stimulate the production of inositol phosphates in HEK $G\alpha_{15}$ cells transiently transfected with the M_4 receptor (HEK $G\alpha_{15}$ M_4 cells). Figure 4a shows the concentration-response curves of the five agonists tested in the HEK $G\alpha_{15}$ M_4 cells. Carbachol and *S*-aceclidine displayed full agonism with similar potency and E_{max}. 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 E_{max}, whereas *R*-aceclidine exhibited both lower potency and E_{max}. Table 3 lists the E_{max}, EC_{50}, and Hill slope values of agonists for these responses.

In a prior study, we showed that an endogenous M_3 muscarinic receptor elicits a weak phosphoinositide response in the HEK $G\alpha_{15}$ cell (Griffin et al., 2007). Therefore, muscarinic responses measured in HEK $G\alpha_{15}$ cells transiently transfected with the M_4 receptor should represent the sum of M_3 and M_4 responses. To quantify the magnitude of the M_3 component, we measured agonist-stimulated phosphoinositide hydrolysis in HEK $G\alpha_{15}$ cells transfected with an empty pcDNA3.1 vector (HEK $G\alpha_{15}$ null, see Fig. 4b). In general, 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_{max} value. Neither McN-A-343 nor *R*-aceclidine produced measurable concentration-dependent agonism in the HEK $G\alpha_{15}$ null cells. Control experiments with HEK $G\alpha_{15}$ M_4 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 M_4 Responses Elicited through G_i, G_s, and $G\alpha_{15}$. The RA_i values of agonists for eliciting responses through G_i (Fig. 2), G_s (Fig. 3), and $G\alpha_{15}$ (Fig. 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 $G\alpha_{15}$ M_4 and HEK $G\alpha_{15}$ null cells

TABLE 2
Agonist activity for stimulation of cAMP accumulation in CHO M_4 cells previously treated with pertussis toxin
The data are from Fig. 3a. The data represent the mean estimates ± S.E.M. The values in parentheses beneath some of the estimates are the Log mean ± S.E.M.

Agonist	E_{max}[a]	EC_{50}	Hill Slope	RA_i	
				Null	Operational
	%	μ*M*			
Oxotremorine-M	106 ± 3.8	0.54 (−6.27 ± 0.06)	1.25 ± 0.21	11.80 (1.07 ± 0.04)	10.67 (1.03 ± 0.10)
Carbachol	78 ± 3.2	5.3 (−5.28 ± 0.07)	1.26 ± 0.24	1.0 (0.0)	1.0 (0.0)
S-Aceclidine	53 ± 5.7	11.8 (−4.93 ± 0.23)	1[b]	0.39 (−0.41 ± 0.37)	0.45 (−0.35 ± 0.14)

[a] Denotes the maximal stimulation of cAMP accumulation expressed as a percentage of basal cAMP accumulation, which is the amount of accumulation in the presence of forskolin (0.1 μM).
[b] Hill slope constrained to 1.

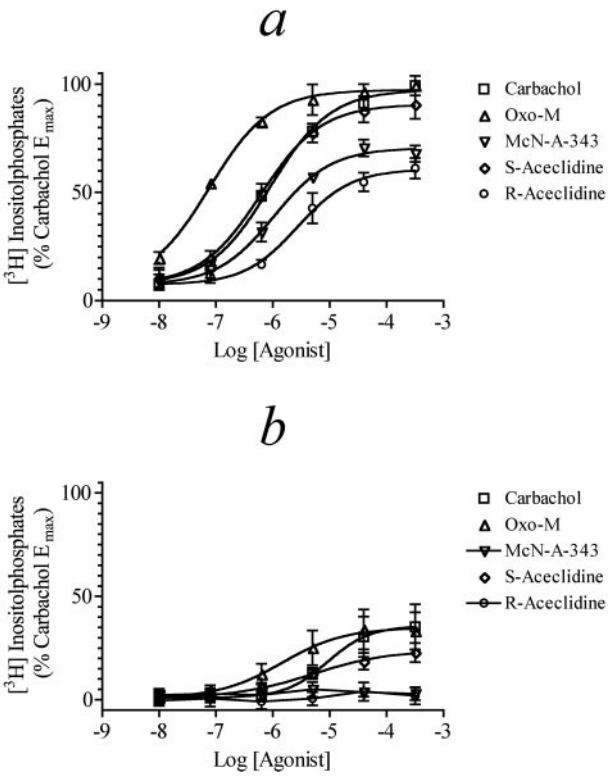

Fig. 4. Muscarinic agonist-mediated phosphoinositide hydrolysis in HEK $G\alpha_{15}$ cells. Agonist-mediated phosphoinositide hydrolysis was measured in HEK $G\alpha_{15}$ M_4 cells (a) and HEK $G\alpha_{15}$ cells (b). The data represent the mean values ± S.E.M. of four experiments, each done in triplicate. The data are expressed relative to the E_{max} for carbachol.

(carbachol and oxo-M). The concentration-response curves from both cell lines were analyzed simultaneously, sharing the estimates of the M_3 parameters between both curves and using only the HEK $G\alpha_{15}$ M_4 cells for estimation of the M_4 parameters. In this manner, the M_4 component of the phosphoinositide response in HEK $G\alpha_{15}$ M_4 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 M_3 response and one for the M_4 response. This careful analysis ultimately showed that the estimate of RA_i value for the M_4 component in the HEK $G\alpha_{15}$ M_4 cells was practically the same as that estimated assuming that the entire response was elicited by the M_4 receptor. Presumably, the endogenous M_3 response was too insensitive to influence the M_4 response significantly.

A summary of the RA_i estimates is shown in Fig. 5, and the corresponding RA_i values are also listed in Tables 1 through 3. Oxo-M exhibited the highest RA_i values, whereas carbachol, *S*-aceclidine, and McN-A-343 all exhibited values similar to each other but somewhat lower than those of oxo-M. *R*-Aceclidine exhibited the lowest RA_i values. None of the agonists exhibited a marked difference in activity for eliciting M_4 responses through the three different G proteins. No RA_i value was calculated for McN-A-343 and *R*-aceclidine in the CHO M_4 G_s assay because of the immeasurable response to these agonists.

Comparison of Agonist Activity across M_1 to M_4 Muscarinic Receptors. We used our RA_i estimates to compare the activity of agonists across the M_1 to M_4 subtypes of the muscarinic receptor. For this analysis, we used data generated from our laboratory in which the test and standard agonists were assayed in the same experiment to minimize variation between experiments. Most of the RA_i estimates for the M_2 receptor were taken from Griffin et al. (2007) in which M_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_3 receptor are from Ehlert et al. (1999). Additional data on the M_1 receptor were obtained and are described below to give a complete picture of activity across the M_1 to M_4 subtypes.

Our data on agonist-mediated stimulation of phosphoinositide hydrolysis in CHO M_1 cells are shown in Fig. 6, a and b. Most of the agonists exhibited a similar maximal response, with the exception of the enantiomers of aceclidine whose E_{max} values were moderately lower. In addition, 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_i Values at M_1 to M_4 Muscarinic Receptors. A summary of the RA_i estimates for agonists across M_1 to M_4 muscarinic receptors is shown in Fig. 7. All values were estimated using the operational method. For this analysis, RA_i values were estimated from phosphoinositide assays in CHO M_1 and CHO M_3 cells and from cAMP assays on CHO M_2 and CHO M_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_1 to M_4 muscarinic subtypes, with an especially high RA_i value of 30 at the M_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_1 to M_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 com-

TABLE 3
Agonist activity for stimulating phosphoinositide hydrolysis in HEK $G\alpha_{15}$ M_4 cells
The data are from Fig. 4. The data represent the mean estimates ± S.E.M. The values in parentheses to the right of some of the estimates are the Log mean ± S.E.M.

Agonist	E_{max}[a]	EC_{50}	Hill Slope	RA_i	
				Null	Operational[b]
	%	μM			
Oxotremorine-M	97 ± 1.1	0.078 (−7.11 ± 0.04)	0.82 ± 0.06	8.01 (0.90 ± 0.04)	10.67 (1.028 ± 0.12)
Carbachol	97 ± 0.9	0.90 (−6.05 ± 0.03)	0.82 ± 0.03	1.0 (0.0)	1.0 (0.0)
McN-A-343	70 ± 1.4	1.1 (−5.98 ± 0.06)	0.87 ± 0.08	0.65 (−0.19 ± 0.07)	0.44 (−0.36 ± 0.05)
S-Aceclidine	91 ± 1.7	0.62 (−6.21 ± 0.05)	0.82 ± 0.07	1.42 (0.15 ± 0.06)	1.24 (0.09 ± 0.06)
R-Aceclidine	61 ± 1.9	2.7 (−5.56 ± 0.08)	0.92 ± 0.13	0.22 (−0.65 ± 0.04)	0.13 (−0.87 ± 0.08

[a] Denotes the maximum stimulation of phosphoinositide hydrolysis by carbachol.
[b] The operational RA_i values for oxotremorine-M and carbachol were estimated using eqs. 10 through 13, whereas those for McN-A-343, *S*-aceclidine, and *R*-aceclidine were estimated using eqs. 8 and 9. In each analysis the concentration-response curve of carbachol was analyzed simultaneously as the standard.

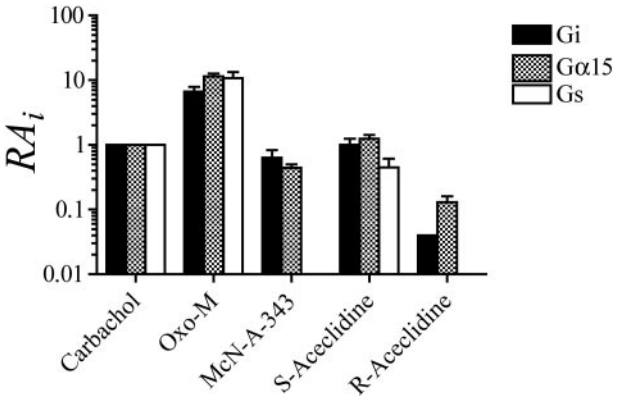

Fig. 5. Comparison of the RA_i values of muscarinic agonists for eliciting different responses through the M_4 receptor via different G proteins. The estimates are from Tables 1 to 3.

pounds were McN-A-343 and pilocarpine. McN-A-343 exhibited high selectivity for the M_1 and M_4 subtypes and much lower activity at the M_2 (0.0020) and M_3 (0.019) subtypes. The RA_i values of McN-A-343 at the M_1 and M_4 subtypes were comparable with those of carbachol. Pilocarpine exhibited activity less than carbachol but showed selectivity between the muscarinic subtypes; its RA_i values for the M_1 (0.49) and M_3 (0.15) subtypes were much higher than those for the M_2 (0.015) and M_4 (0.013) receptors. One way analysis of variance showed no significant differences among the log RA_i values of *S*-aceclidine ($F_{3,12} = 1.16$; $P = 0.37$) and *R*-aceclidine ($F_{3,12} = 2.40$; $P = 0.12$) across the M_1 to M_4 receptor subtypes. In contrast, oxo-M ($F_{3,12} = 43.29$; $P = 1.03 \times 10^{-6}$), McN-A-343 ($F_{3,12} = 105.9$; $P = 8.6 \times 10^{-10}$), pilocarpine ($F_{3,10} = 18.30$; $P = 2.2 \times 10^{-4}$), and arecoline ($F_{3,10} = 4.76$ $P = 0.0260$) exhibited significant differences in their log RA_i values across receptor subtypes. Post hoc comparisons using T tests with the Bonferroni adjustment showed that oxo-M exhibited selectivity for M_1 receptors relative to M_2 to 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 RA_i values of arecoline at the M_1 to M_4 subtypes.

Estimation of RA_i Values from Published Data. Because the estimation of RA_i only requires the agonist concentration-response curve, it should be possible to estimate RA_i 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 RA_i 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 van Giersbergen (1995) (CHO M_1), Schwarz et al. (1993) (CHO M_1), and Mei et al. (1991) (B82 fibroblasts transfected with the M_1 receptor); agonist-stimulated GTPase activity in CHO M_1 cells (Lazareno and Birdsall, 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. However, there is some question 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 RA_i values. The studies on cell lines included experiments on the inhibition of cAMP accumulation in CHO M_2 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 Mitchelson (1997) and Lambrecht et al. (1993). RA_i values for the M_3 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 and Nahorski (1988) (parotid gland and ileum), Matsumoto et al. (1994) (ciliary muscle), and Ehlert et al. (1999) (CHO M_3 cells). The data on the contractility of the ileum was from Ringdahl et al. (1982), Hanin et al. (1966), and Ehlert et al. (1999). RA_i values were calculated as described under *Materials and Methods* and plotted as scatter plots for comparison in Fig. 8. We have indicated those values that were calculated from the rabbit vas deferens with an asterisk because this tentative M_1 response may actually be an M_4 response. If the E_{max} of the standard and reference agonist were the same, the RA_i would be estimated as the potency ratio (see Griffin et al., 2007).

The greatest variation in RA_i values was noted at the M_2 receptor (standard deviation of Log $RA_i = 0.45$; 2.8-fold), the least variation at the M_3 receptor (standard deviation of Log $RA_i = 0.14$; 1.4-fold), and intermediate variation at the M_1 receptor (standard deviation of Log $RA_i = 0.39$; 2.5-fold). One-way analysis of variance revealed no significant differ-

TABLE 4

Agonist activity for stimulating phosphoinositide hydrolysis in CHO M_1 cells

The data are from Fig. 6, a and b. The data represent the mean estimates ± S.E.M. The values in parentheses to the right of some of the estimates are the Log mean ± S.E.M.

Agonist	E_{max}[a]	EC_{50}	Hill Slope	RA_i	
				Null	Operational
	%	μM			
Oxotremorine-M	100 ± 2.3	0.041 (−7.39 ± 0.05)	1.16 ± 0.13	32 (1.51 ± 0.28)	30 (1.48 ± 0.04)
Carbachol	98 ± 1.1	1.4 (−5.86 ± 0.02)	1.29 ± 0.07	1.0 (0.0)	1.0 (0.0)
McN-A-343	77 ± 1.7	1.9 (−5.72 ± 0.04)	1.09 ± 0.09	0.69 (−0.16 ± 0.06)	0.64 (−0.20 ± 0.07)
S-Aceclidine	102 ± 1.5	3.8 (−5.42 ± 0.03)	1.29 ± 0.07	0.72 (−0.14 ± 0.11)	0.66 (−0.18 ± 0.04)
R-Aceclidine	61 ± 0.46	3.1 (−5.51 ± 0.01)	1.25 ± 0.03	0.31 (−0.51 ± 0.11)	0.22 (−0.66 ± 0.04)
Arecoline	118 ± 2.9	1.7 (−5.77 ± 0.05)	0.93 ± 0.08	1.51 (0.18 ± 0.04)	1.22 (0.09 ± 0.04)
Pilocarpine	119 ± 3.1	5.7 (−5.24 ± 0.05)	0.81 ± 0.06	0.48 (−0.32 ± 0.06)	0.49 (−0.31 ± 0.04)

[a] Denotes the maximal stimulation of phosphoinositide hydrolysis by carbachol.

TABLE 5

Agonist activity for inhibiting forskolin-stimulated cAMP accumulation in CHO M_2 cells

The data represent the mean estimates ± S.E.M. The values in parentheses to the right of some estimates are the Log mean ± S.E.M. NC denotes not calculated because the E_{max} for the test agonist was the same as the standard carbachol; therefore, RA_i values were calculated as the potency ratio.

Agonist	E_{max}[a]	EC_{50}	Hill Slope	RA_i	
				Null	Operational
	%	μM			
Oxotremorine-M[b]	73 ± 2.3	0.047 (−7.32 ± 0.07)	1.02 ± 0.06	NC	4.7 (0.67 ± 0.03)
Carbachol[b]	73 ± 2.3	0.22 (−6.65 ± 0.07)	0.90 ± 0.08	1.0 (0.0)	1.0 (0.0)
McN-A-343[b]	32 ± 5.1	38 (−4.42 ± 0.13)	1.17 ± 0.28	0.0022 (−2.66 ± 0.23)	0.0024 (−2.62 ± 0.12)
S-Aceclidine[b]	73 ± 2.3	0.41 (−6.39 ± 0.10)	0.89 ± 0.07	NC	0.55 (−0.26 ± 0.06)
R-Aceclidine[b]	73 ± 2.3	2.6 (−5.59 ± 0.08)	0.83 ± 0.08	NC	0.087 (−1.06 ± 0.04)
Arecoline	45 ± 1.9	0.83 (−6.08 ± 0.07)	1.20 ± 0.16	0.43 (−0.36 ± 0.05)	0.35 (−0.45 ± 0.08)
Pilocarpine	40 ± 1.4	17 (−4.76 ± 0.06)	1.11 ± 0.16	0.014 (−1.86 ± 0.03)	0.015 (−1.85 ± 0.07)

[a] Denotes the maximal inhibition of forskolin-stimulated cAMP accumulation.
[b] Data are from Griffin et al., 2007.

ences in the Log RA_i values of oxotremorine ($F_{2,6} = 2.31$; $P = 0.18$), *S*-aceclidine ($F_{2,5} = 3.77$; $P = 0.10$), *R*-aceclidine ($F_{2,5} = 1.93$; $P = 0.24$), and arecoline ($F_{2,11} = 1.26$; $P = 0.32$) at the M_1 to M_3 subtypes. In contrast, oxo-M ($F_{2,11} = 5.78$; $P = 0.017$), McN-A-343 ($F_{2,10} = 43.69$; $P = 1.14 \times 10^{-5}$), and pilocarpine ($F_{2,9} = 9.81$; $P = 0.005$) exhibited significant differences at the M_1 to M_3 subtypes. Oxo-M had geometric mean RA_i values of 15.5, 8.6, and 3.0 at the M_1, M_2, and M_3 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 RA_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 RA_i values for pilocarpine at the M_1 to 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 RA_i values for an agonist within the same receptor type highlights differences between studies. RA_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 RA_i estimates at the M_1 receptor is also seen for oxotremorine, with RA_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 Fig. 8, the RA_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, and 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 RA_i values between the M_1 studies evaluated.

The RA_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 RA_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 RA_i estimates of all compounds illustrated in Fig. 8, with a standard deviation of log RA_i values of 0.21. As described above, the variance in agonist RA_i values is substantially decreased when surveying M_3-based assays.

Discussion

The RA_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, different RA_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_4 receptor coupling to G_i, G_s, or $G\alpha_{15}$. Our data with the M_4 receptor provide no evidence for different active states of the M_4 receptor. This result may suggest that measurement of M_4 activation via $G\alpha_{15}$ is an appropriate substitute for estimating agonist activity at the M_4 receptor signaling through G_i, but it is conceivable that other novel agonists may preferentially direct signaling at the M_4 receptor through one G protein more than another. For example, at the M_2 receptor, it has been shown that McN-A-343 has 10-fold greater activity when activating M_2 receptor signaling via $G\alpha_{15}$ versus G_i (Griffin et al., 2007). Therefore, before

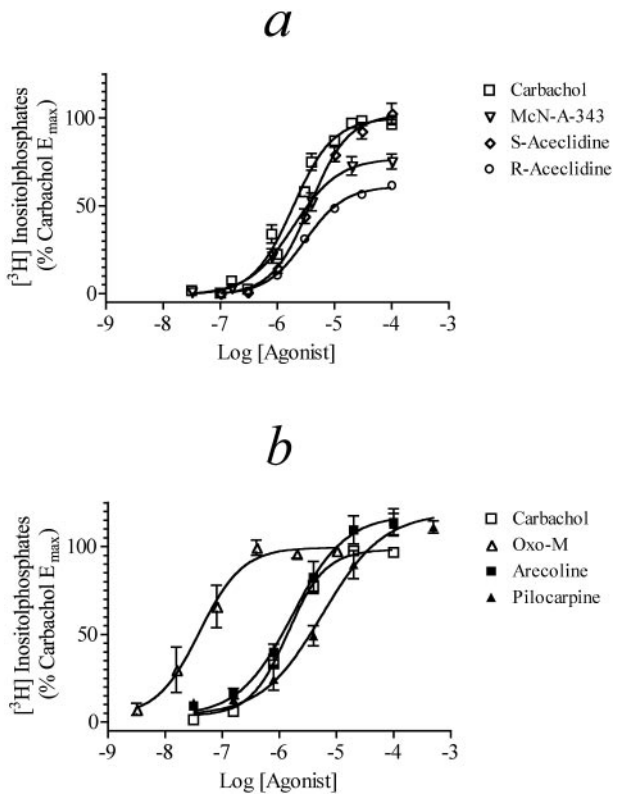

Fig. 6. Muscarinic agonist-mediated phosphoinositide hydrolysis in M_1 CHO cells. Agonist-mediated phosphoinositide hydrolysis was measured in CHO cells stably transfected with the human M_1 receptor. Concentration-response curves are shown for carbachol, McN-A-343, *S*-aceclidine, and *R*-aceclidine (a) and carbachol, oxo-M, arecoline, and pilocarpine (b). Mean values ± S.E.M. of three experiments are shown with each done in triplicate. The data are expressed relative to the E_{max} for carbachol.

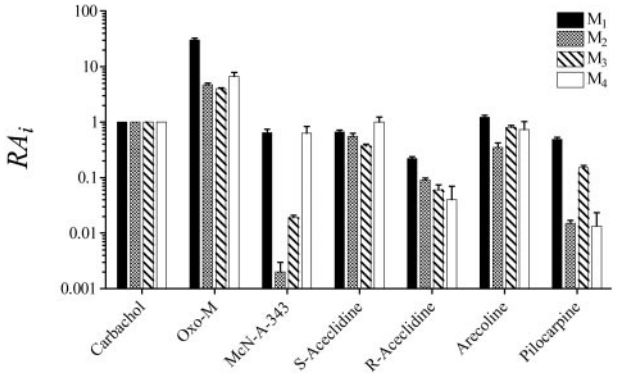

Fig. 7. Comparison of the RA_i values of agonists for eliciting responses in CHO cells transfected with M_1 to M_4 muscarinic receptors. The estimates are from Tables 1, 4, and 5 and Ehlert et al. (1999).

implementation of a cellular screen based on alternative G protein signaling, it would seem prudent to use RA_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_4 signaling through G_s were much higher than those required to inhibit forskolin-stimulated cAMP via M_4 signaling through G_i, suggesting a possible physiological irrelevance of the M_4 activation via G_s. Nonetheless, this pathway does provide an additional example of the use of RA_i in alternative screening paradigms.

Because the RA_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_1 to M_4 subtypes requiring the two parameters, EC_{50} and E_{max}. RA_i also presents an advantage over the use of potency ratios because RA_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 M_1 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 M_1 cells by Schwarz et al. (1993) (87 and 66%, respectively) and Richards and van Giersbergen (1995) (85 and 76%, respectively).

In this study, McN-A-343 displayed increased RA_i values at the M_1 and M_4 receptors compared with M_2 and M_3. This pattern correlates with previous data indicating selectivity of McN-A-343 for both M_1 and M_4 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 (M_1) 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 M_1-selective antagonist pirenzepine (Eltze, 1988). It is conceivable that this response is mediated by the M_4 receptor because pirenzepine exhibits moderately high affinity for the M_4 receptor (pK_D = 7.23) in addition to its high affinity for the M_1 receptor (pK_D = 7.77) (Ehlert et al., 1997). In cell lines, McN-A-343 exhibits greater potency and maximal effect at stimulating GTPase activity in CHO M_4 cells, compared with that observed in CHO M_1 cells, but exhibits much lower activity at the M_2 and M_3 subtypes (Lazareno et al., 1993).

Pilocarpine exhibited RA_i values of 0.49, 0.015, 0.21, and 0.01 across M_1 to M_4 receptors, respectively, indicating selectivity for M_1 and M_3 receptors compared with M_2 and M_4. Pilocarpine has previously been shown to exhibit selectivity for the M_1 receptor based on its activation of GTPase activity in CHO M_1 cells (Lazareno and Birdsall, 1993). More recently, Fox et al. (2001) showed that the salivating effect of pilocarpine is due to its selective stimulation of M_1 and M_3 receptors present on salivary glands, and Gautam et al. (2004) described how the salivary effect of pilocarpine is prevented in M_1/M_3 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_1 and M_3 receptors in this tissue. Our demonstration of the M_1 and M_3 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_1 receptor.

The final section of this report compared RA_i 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_i values for a compound, it may indicate a differ-

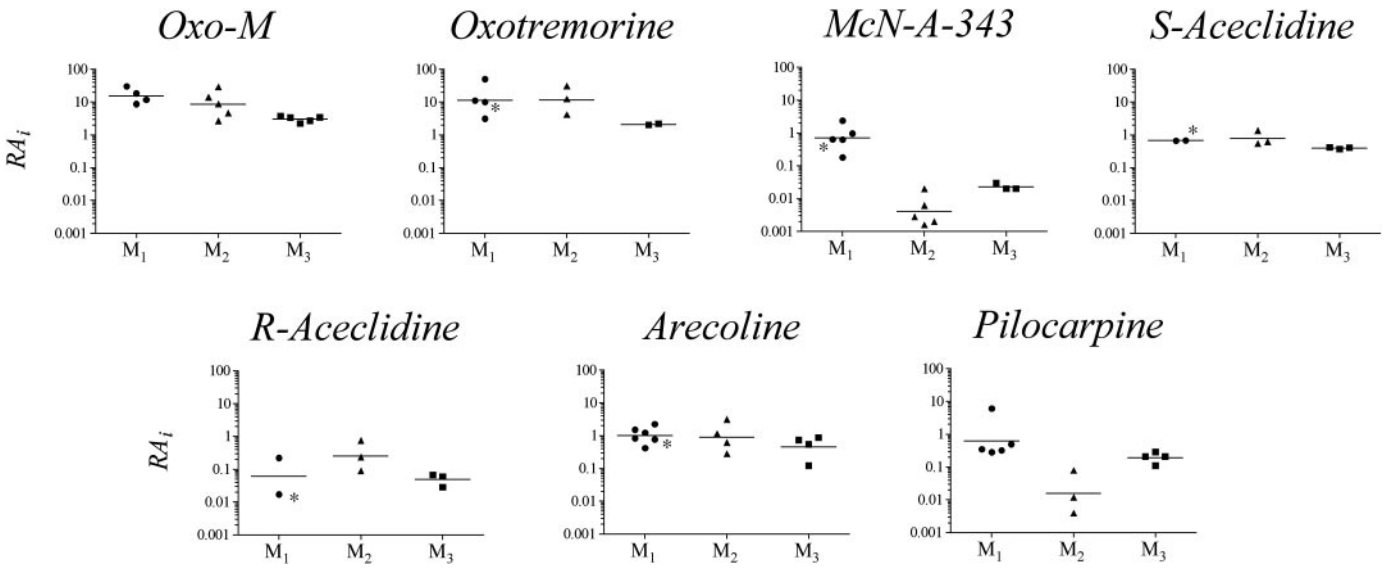

Fig. 8. Comparison of the RA_i values of agonists for eliciting different responses in assays for M_1, M_2, and M_3 muscarinic receptors. The estimates were calculated from the published concentration-response curves of Lazareno et al. (1993), Eltze et al. (1993), Mei et al. (1991), Richards and van 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 (1997), Ehlert et al. (1999), Matsumoto et al. (1994), Ek and Nahorski (1998), Hanin et al. (1966) and Ringdahl et al. (1982), Wang and El-Fakahany (1993), and Lambrecht et al. (1993). Asterisks are used to indicate the RA_i values estimated from the study of Eltze et al. (1993) on the rabbit vas deferens because there is a question whether this response is M_1 (as indicated in the figure) or M_4.

TABLE 6

Rank order of agonist activity based upon RA_i values calculated via the operational method

Data were taken from Tables 1, 4, and 5 and Fig. 7.

Receptor	RAi Rank Order
M_1	Oxo-M > >arecoline = carbachol > *S*-aceclidine = McN-A-343 > pilocarpine > *R*-aceclidine
M_2	Oxo-M > carbachol > *S*-aceclidine > arecoline > *R*-aceclidine > pilocarpine > McN-A-343
M_3	Oxo-M > carbachol = arecoline > *S*-aceclidine > pilocarpine > *R*-aceclidine = McN-A-343
M_4	Oxo-M > carbachol = *S*-aceclidine > arecoline > McN-A-343 > *R*-aceclidine = pilocarpine

ence 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 interassay variability was adequate. There is a distinct lack of variation in the estimates of RA_i by different investigators for agonist activity at the M_3 receptor (Fig. 7). With the exception of the RA_i of arecoline in the ciliary muscle of the rabbit (Matsumoto et al., 1994), all agonists presented very similar RA_i 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, 1999). Variability in agonist activity across assays was evident in the M_1 and M_2 sets of data. At the M_1 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_1 cells; however, Lazareno et al. (1993) measured GTPase activity, whereas Schwarz et al. (1993) measured phosphoinositide hydrolysis. The high RA_i 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, with the exception of oxo-M, tested in the study of Lazareno et al. (1993) are higher than those calculated from other M_1-based studies, suggesting unusually low activity for carbachol. The pattern of selectivity that we observed in our studies (Fig. 6) is generally consistent with data from the literature on the M_1 to M_3 subtypes (Fig. 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 10-fold lower than that calculated here in the CHO M_1 cells. *R*-Aceclidine is a good substrate for acetylcholinesterase (Pyttel and Robinson, 1973). Therefore, its activity 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 is 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, the RA_i estimate represents a weighted average, depending on the receptor conformations selected by the ligand and attendant effectors (e.g., G proteins). Al-

though 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 Fig. 4, making RA_i analysis a powerful tool for quantifying ligand-directed signaling.

Appendix

This appendix describes the derivation of eqs. 10 through 13, which were used for the analysis of the concentration-response curves in HEK $G\alpha_{15}$ M_4 and HEK $G\alpha_{15}$ 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).

$$\text{Response} = \frac{s^N M}{s^N + K_E^N} \tag{15}$$

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.

$$\text{Response} = \frac{M}{1 + \frac{1}{(s/K_E)^N}} \tag{16}$$

Substituting in a parameter (S) for s/K_E yields eqs. 10 and 11 under *Materials and Methods*. The stimulus is defined according to Furchgott (1966).

$$s = \frac{X\varepsilon R_T}{X + K} \tag{17}$$

In this equation, X denotes the concentration of agonist, ε 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 eq. 17 by K_E yields

$$S = \frac{X\tau}{X + K} \tag{18}$$

in which

$$\tau = \frac{\varepsilon R_T}{K_E} \tag{19}$$

Equations 18 and 19 provide the basis for eqs. 12 and 13 under *Materials and Methods*.

References

Berridge MJ, Downes CP, and Hanley MR (1982) Lithium amplifies agonist-dependent phosphatidylinositol responses in brain and salivary glands. *Biochem J* **206:**587–595.

Black JW and Leff P (1983) Operational models of pharmacological agonism. *Proc R Soc Lond B Biol Sci* **220:**141–162.

Buckley NJ and Burnstock G (1986) Autoradiographic localization of peripheral M_1 muscarinic receptors using [^{3}H]pirenzepine. *Brain Res* **375:**83–91.

Bymaster FP, Carter PA, Yamada M, Gomeza J, Wess J, Hamilton SE, Nathanson NM, McKinzie DL, and Felder CC (2003) Role of specific muscarinic receptor subtypes in cholinergic parasympathomimetic responses, in vivo phosphoinositide hydrolysis, and pilocarpine-induced seizure activity. *Eur J Neurosci* **17:**1403–1410.

Christopoulos A and Mitchelson F (1997) Pharmacological analysis of the mode of interaction of McN-A-343 at atrial muscarinic M_2 receptors. *Eur J Pharmacol* **339:**153–156.

Colquhoun D (1998) Binding, gating, affinity and efficacy: the interpretation of structure-activity relationships for agonists and of the effects of mutating receptors. *Br J Pharmacol* **125:**924–947.

Ehlert FJ (2008) On the analysis of ligand-directed signaling at G protein-coupled receptors. *Naunyn Schmiedebergs Arch Pharmacol* **377:**549–577.

Ehlert FJ (1985) The relationship between muscarinic receptor occupancy and adenylate cyclase inhibition in the rabbit myocardium. *Mol Pharmacol* **28:**410–421.

Ehlert FJ (2000) Ternary complex model, in *Biomedical Applications of Computer Modeling* (Christopoulos A ed) pp 21–85, CRC Press, Boca Raton.

Ehlert FJ, Griffin MT, and Glidden PF (1996) The interaction of the enantiomers of aceclidine with subtypes of the muscarinic receptor. *J Pharmacol Exp Ther* **279:**1335–1344.

Ehlert FJ, Griffin MT, Sawyer GW, and Bailon R (1999) A simple method for estimation of agonist activity at receptor subtypes: comparison of native and cloned M_3 muscarinic receptors in guinea pig ileum and transfected cells. *J Pharmacol Exp Ther* **289:**981–992.

Ehlert FJ, Thomas EA, Gerstin EH, and Griffin MT (1997) Muscarinic receptors and gastrointestinal smooth muscle, in *Muscarinic Receptor Subtypes in Smooth Muscle* (Eglen RM ed) pp 92–147, CRC Press, Boca Raton.

Ek B and Nahorski S (1988) Muscarinic receptor coupling to inositol phospholipid metabolism in guinea-pig cerebral cortex, parotid gland and ileal smooth muscle. *Biochem Pharmacol* **37:**4461–4467.

Eltze M (1988) Muscarinic M_1- and M_2-receptors mediating opposite effects on neuromuscular transmission in rabbit vas deferens. *Eur J Pharmacol* **151:**205–221.

Eltze M, Ullrich B, Mutschler E, Moser U, Bungardt E, Friebe T, Gubitz C, Tacke R, and Lambrecht G (1993) Characterization of muscarinic receptors mediating vasodilation in rat perfused kidney. *Eur J Pharmacol* **238:**343–355.

Fechner PU, Teichmann KD, and Weyrauch W (1975) Accommodative effects of aceclidine in the treatment of glaucoma. *Am J Ophthalmol* **79:**104–106.

Fisher SK and Bartus RT (1985) Regional differences in the coupling of muscarinic receptors to inositol phospholipid hydrolysis in guinea pig brain. *J Neurochem* **45:**1085–1095.

Fox RI, Konttinen Y, and Fisher A (2001) Use of Muscarinic Agonists in the Treatment of Sjögren's Syndrome. *Clin Immunol* **101:**249–263.

Furchgott RF (1966) The use of β-haloalkylamines in the differentiation of receptors and in the determination of dissociation constants of receptor-agonist complexes. *Adv Drug Res* **3:**21–55.

Gautam D, Heard TS, Cui Y, Miller G, Bloodworth L, and Wess J (2004) Cholinergic stimulation of salivary secretion studied with M_1 and M_3 muscarinic receptor single- and double-knockout mice. *Mol Pharmacol* **66:**260–267.

Griffin MT, Figueroa KW, Liller S, and Ehlert FJ (2007) Estimation of agonist activity at G protein-coupled receptors: analysis of M_2 muscarinic receptor signaling through $G_{i/o}$, G_s, and G_q. *J Pharmacol Exp Ther* **321:**1193–1207.

Hammer R, Berrie CP, Birdsall NJ, Burgen AS, and Hulme EC (1980) Pirenzepine distinguishes between different subclasses of muscarinic receptors. *Nature* **283:**90–92.

Hanin I, Jenden DJ, and Cho AK (1966) The influence of pH on the muscarinic action of oxotremorine, arecoline, pilocarpine, and their quaternary ammonium analogs. *Mol Pharmacol* **2:**352–359.

Keen M and Nahorski SR (1988) Muscarinic acetylcholine receptors linked to the inhibition of adenylate cyclase activity in membranes from the rat striatum and myocardium can be distinguished on the basis of agonist efficacy. *Mol Pharmacol* **34:**769–778.

Kenakin T (2007) Collateral efficacy in drug discovery: taking advantage of the good (allosteric) nature of 7TM receptors. *Trends Pharmacol Sci* **28:**407–415.

Kendall D and Hill S (1990) Measurement of [^{3}H]inositol phospholipid turnover, in *Methods in Neurotransmitter Receptor Analysis* (Yamamura H ed) pp 68–87, Raven Press, New York.

Lambrecht G, Moser U, Grimm U, Pfaff O, Hermanni U, Hildebrandt C, Waelbroeck M, Christophe J, and Mutschler E (1993) New functionally selective muscarinic agonists. *Life Sci* **52:**481–488.

Lazareno S and Birdsall NJ (1993) Pharmacological characterization of acetylcholin [^{35}S]-GTPγS binding mediated by human muscarinic m1–m4 receptors: antagonist studies. *Br J Pharmacol* **109:**1120–1127.

Matsumoto S, Yorio T, DeSantis L, and Pang IH (1994) Muscarinic effects on cellular functions in cultured human ciliary muscle cells. *Invest Ophthalmol Vis Sci* **35:**3732–3738.

McKinney M, Miller JH, Gibson VA, Nickelson L, and Aksoy S (1991) Interactions of agonists with M_2 and M_4 muscarinic receptor subtypes mediating cyclic AMP inhibition. *Mol Pharmacol* **40:**1014–1022.

Mei L, Lai J, Yamamura HI, and Roeske WR (1991) Pharmacologic comparison of selected agonists for the M1 muscarinic receptor in transfected murine fibroblast cells (B82). *J Pharmacol Exp Ther* **256:**689–694.

Michal P, El-Fakahany EE, and Dolezal V (2007) Muscarinic M_2 receptors directly activate G_q/11 and G_s G-proteins. *J Pharmacol Exp Ther* **320:**607–614.

Milligan G and Kostenis E (2006) Heterotrimeric G-proteins: a short history. *Br J Pharmacol* **147 (Suppl. 1):**S46–S55.

Mistry R, Dowling MR, and Challiss RAJ (2005) An investigation of whether agonist-selective receptor conformations occur with respect to M_2 and M_4 muscarinic acetylcholine receptor signalling via Gi/o and Gs proteins. *Br J Pharmacol* **144:**566–575.

Offermanns S, Negulescu P, Hu YH, and Simon MI (2001) Conditionally expressed

Ga15 couples to endogenous receptors in GH3 cells. *Naunyn Schmiedebergs Arch Pharmacol* **364:**140–148.
Pyttel R and Robinson JB (1973) Interaction of 3-quinuclidinol and its derivatives with acetylcholinesterase. *J Pharm Sci* **62:**684–685.
Richards MH and van Giersbergen PL (1995) Human muscarinic receptors expressed in A9L and CHO cells: activation by full and partial agonists. *Br J Pharmacol* **114:**1241–1249.
Ringdahl B, Ehlert FJ, and Jenden DJ (1982) Muscarinic activity and receptor binding of the enantiomers of aceclidine and its. *Mol Pharmacol* **21:**594–599.
Ringdahl B, Resul B, and Dahlbom R (1979) Facile preparation of the enantiomers of 3-acetoxyquinuclidine and 3-quinuclidinol. *Acta Pharm Suec* **16:**281–283.
Roszkowski AP (1961) An unusual type of sympathetic ganglionic stimulant. *J Pharmacol Exp Ther* **132:**156–170.
Schwarz RD, Davis RE, Jaen JC, Spencer CJ, Tecle H, and Thomas AJ (1993) Characterization of muscarinic agonists in recombinant cell lines. *Life Sci* **52:**465–472.
Wang SZ and El-Fakahany EE (1993) Application of transfected cell lines in studies of functional receptor subtype selectivity of muscarinic agonists. *J Pharmacol Exp Ther* **266:**237–243.

Address correspondence to: Dr. Frederick J. Ehlert, Department of Pharmacology, University of California, Irvine, Irvine, California 92697-4625. E-mail: fjehlert@uci.edu