Estimation of Agonist Activity at G Protein-Coupled Receptors: Analysis of M2 Muscarinic Receptor Signaling through $G_{i/o}$, $G_s$, and $G_{15}$

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ABSTRACT

We developed novel methods for analyzing the concentration-response curve of an agonist to estimate the product of observed affinity and intrinsic efficacy, expressed relative to that of a standard agonist. This parameter, termed intrinsic relative activity ($RA_i$), is most applicable for the analysis of responses at G protein-coupled receptors. $RA_i$, equivalent to the potency ratios that agonists would exhibit in a hypothetical, highly sensitive assay in which all agonists behave as full agonists, even those with little intrinsic efficacy. We investigated muscarinic responses at the M$_2$ receptor, including stimulation of phosphoinositide hydrolysis through $G_{s}$ in HEK 293T cells, inhibition of cAMP accumulation through $G_i$ in Chinese hamster ovary (CHO) cells, and stimulation of cAMP accumulation through $G_s$ in CHO cells treated with pertussis toxin. The $RA_i$ values of carbachol, oxotremorine-M, and the enantiomers of aceclidine were approximately the same in the three assay systems. In contrast, the activity of 4-[[N-[3-chlorophenyl]carbamoy]oxy-2-butynyl]trimethylammonium chloride (McN-A-343) was ~10-fold greater at M$_2$ receptors coupled to $G_{s}$ in HEK 293T cells compared with M$_2$ receptors coupled to $G_i$ in the same cells or in CHO cells. Our results show that the $RA_i$ estimate is a useful measure for quantifying agonist activity across different assay systems and for detecting agonist directed signaling.

Drug discovery often involves testing compounds in isolated tissues and high throughput assays to determine activity at target receptors. In the case of agonists, the measured parameters are usually $EC_{50}$ and $E_{\text{max}}$, whereas the parameters of greater relevance to drug design are observed affinity and intrinsic efficacy. If the receptor is a ligand-gated ion channel, then receptor activation can be measured directly as whole cell current, and the $EC_{50}$ and $E_{\text{max}}$ are reasonable estimates of observed affinity and intrinsic efficacy provided that desensitization is not excessive. It would seem that, in most instances, the observed affinity and intrinsic efficacy of an agonist for a ligand-gated ion channel would be constant, regardless of the tissue or cell in which the receptor is expressed.

The situation is more complex for a G protein-coupled receptor (GPCR). First, GPCRs are inactive in isolation and must interact with a G protein to elicit a response. It is possible that the G protein with which the receptor interacts selects for a receptor conformation having a unique agonist profile and that the observed affinity and intrinsic efficacy of the agonist-receptor complex may be G protein-specific or influenced by other proteins interacting with the receptor (Leff et al., 1997; Berg et al., 1998). Second, because it is difficult to measure receptor activation directly, most assays involve measuring a downstream response, and the corresponding $EC_{50}$ and $E_{\text{max}}$ values may vary, depending on the point in the signaling cascade at which the experimenter measures the response. Therefore, the magnitude of the response elicited by a GPCR is usually not proportional to receptor activation, and $EC_{50}$ and $E_{\text{max}}$ are not equivalent to observed affinity and intrinsic efficacy. Rather, $EC_{50}$ and $E_{\text{max}}$...
$E_{\text{max}}$ depend on the receptor, proteins interacting with the receptor, and a long list of downstream elements that affect the sensitivity of the response. Because the number of non-receptor entities that affect observed affinity and intrinsic efficacy is small compared with the long list of cellular components affecting $EC_{50}$ and $E_{\text{max}}$, it would seem that observed affinity and intrinsic efficacy would be useful parameters for the characterization of the agonist-GPCR complex across different assays. The estimation of these parameters, however, requires additional data not obtained in high throughput screens. Fortunately, it is possible to estimate the product of observed affinity and intrinsic efficacy of an agonist relative to that of a standard agonist just through analysis of the concentration-response curves (Ehlert et al., 1988; Ehler and Griffin, 2001). This product is equivalent to the potency ratio that an agonist would exhibit relative to a standard agonist in a hypothetical, highly sensitive assay in which both agonists behave as full agonists, even if one or both have very little observed intrinsic efficacy. When viewed from this perspective, estimation of the product of observed affinity and efficacy provides a means of converting potential differences in both the $EC_{50}$ and $E_{\text{max}}$ values of agonists into a single, relative measurement, analogous to the potency ratio. Therefore, the product of observed affinity and intrinsic efficacy should be a useful, receptor-dependent parameter for measuring agonist activity at GPCRs.

Here we describe methods for analyzing agonist concentration-response curves to estimate the product of observed affinity and intrinsic efficacy expressed relative to that of a standard agonist. We refer to this product as intrinsic relative activity ($RA_i$). The calculations are the same as those used in the analysis of allosterism in which the control agonist concentration-response curve is compared with one whose properties of affinity and efficacy have been modified allosterically (Ehler, 1988, 2005). This situation is analogous to comparing the concentration-response curves of two different agonists with differing affinities and efficacies. We have estimated the $RA_i$ values of a group of agonists for eliciting responses through the human $M_2$ receptor coupled to $G_i$, $G_{15}$, and $G_o$ to determine whether the $G$ protein with which the $M_2$ receptor interacts influences the activity of the selected agonists. Although most of the agonists investigated exhibited similar $RA_i$ values for triggering $M_2$ muscarinic responses through the different $G$ proteins, compound McN-A-343 exhibited ~10-fold greater activity when stimulating $M_2$-$G_{15}$ responses compared with $M_2$-$G_o$ responses. Our results show that $RA_i$ is a useful parameter for the estimation of agonist activity across different assays.

### Materials and Methods

#### Cell Culture

Chinese hamster ovary (CHO) cells stably expressing the human $M_2$ muscarinic receptor (CHO $M_2$ cells) were obtained from Acadia Pharmaceuticals (San Diego, CA) and cultured in Dulbecco's modified Eagle's medium with high glucose plus l-glutamine (DMEM) supplemented with 10% fetal calf serum, penicillin-streptomycin (100 units/ml) and G 418 (0.4 mg/ml) and puromycin (0.625 μg/ml). A plasmid containing the human $M_2$ receptor (h$M_2$ pCD clone) was kindly provided by Dr. Tom Bonner (National Institutes of Health, Bethesda, MD) and subcloned into a hygromycin(−)pcDNA3.1 vector as described previously (Griffin et al., 2003). HEK $G_{15}$ cells were transiently transfected with the human $M_2$ receptor (HEK $G_{15}$ $M_2$ cells) using Lipofectamine 2000 and 15 μg of the $M_2$ vector. HEK 293T cells were stably transfected with the human $M_2$ receptor in the same manner, and clones of stably transfected cells (HEK $M_2$) were isolated in DMEM containing 10% fetal calf serum, penicillin-streptomycin (100 units/ml), hygromycin (0.2 mg/ml), and G 418 (0.4 mg/ml).

#### cAMP Accumulation

The effects of muscarinic agonists on forskolin- or isoproterenol-stimulated cAMP accumulation was measured in CHO $M_2$ and HEK $M_2$ cells using a modification of the $[\text{3H}]$adenine prelabeling method of Schultz et al. (1972) and the chromatography procedure of Salomon et al. (1974). Confluent cell monolayers grown in T75 flasks were washed with DMEM media and then incubated in 9 ml of DMEM containing $[\text{3H}]$adenine (60 μCi) and adenine (3 μM) for 1 h at 37°C in 5% CO$_2$. Cells were washed twice with DMEM and harvested using trypsin. The resulting cell suspensions were centrifuged for 10 min at 350g, suspended in Krebs-Ringer bicarbonate (KRB) buffer (124 mM NaCl, 5 mM KCl, 1.3 mM MgCl$_2$, 26 mM NaHCO$_3$, 1.2 mM KH$_2$PO$_4$, 1.8 mM CaCl$_2$, and 10 mM glucose) at pH 7.4 and centrifuged a second time. Ultimately, cells were suspended in KRB buffer (12 ml) containing isobutylmethylxanthine (0.5 mM) and incubated for an additional 10 min at 37°C before use in the cAMP assay. Muscarinic agonist-mediated inhibition of cAMP accumulation was carried out in plastic tubes containing intact cells, forskolin (10 μM), isobutylmethylxanthine (0.5 mM), and various concentrations of a muscarinic agonist in a final volume of 0.35 ml of KRB buffer. The reaction was started by the addition of an aliquot (300 μl) of cells and was stopped by addition of an aliquot (200 μl) of ice-cold 30% (w/v) trichloroacetic acid. After at least 30 min on ice, the tubes were centrifuged for 10 min at 3000g, and an aliquot (0.5 ml) from each tube was applied to a Dowex 1.5-column (AG-50W\-X4, 200–400 mesh) and washed with two aliquots of water (1.25 ml each) to remove $[\text{3H}]$ATP. The $[\text{3H}]$cAMP was eluted onto a column of neutral alumina (0.6 g) with 4 ml of water and then eluted into scintillation vials with 4 ml of 0.1 M imidazole HCl (pH 7.5). The samples were counted by liquid scintillation spectroscopy. In experiments in which muscarinic agonist-mediated stimulation in cAMP accumulation was measured, cells were first treated overnight (16–18 h) with pertussis toxin (0.1 μg/ml; final concentration) before labeling with $[\text{3H}]$adenine.

#### Phosphoinositide Hydrolysis

Muscarinic agonist-mediated stimulation of phosphoinositide hydrolysis was measured in suspensions of HEK $G_{15}$ and HEK $G_{15}$ $M_2$ cells using a modification of the $[\text{3H}]$inositol prelabeling method of Berridge et al. (1982) and the extraction method of Kendall and Hill (1990). Confluent cell monolayers grown in T75 flasks were washed with DMEM and then incubated in 9 ml of DMEM containing $[\text{3H}]$inositol (45 μCi) for 16 to 18 h at 37°C in 5% CO$_2$. Cells were washed twice with DMEM and harvested using trypsin as described above. Ultimately, the cell pellet was suspended in KRB buffer (15 ml) containing LiCl (10 mM) and incubated at 37°C for 15 min. Muscarinic agonist-mediated stimulation of $[\text{3H}]$inositol phosphate accumulation was performed in plastic tubes containing intact cells, LiCl (10 mM), and various concentrations of muscarinic agonist in a final volume of 333 μl of KRB buffer. The reaction was started with the addition of an aliquot (300 μl) of cell suspension, and the mixture was gassed with O$_2$-CO$_2$ bubbled with a rubber stopper, and incubated at 37°C for 15 min. The reaction was stopped with 5% perchloric acid (200 μl), and the tubes were placed on ice. $[\text{3H}]$Inositol phosphates were isolated as described previously (Tran et al., 2006). In some experiments, cells were treated with pertussis toxin (0.1

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μg/ml final concentration) overnight during the incubation with [3H]inositol. In experiments using 4-DAMP mustard, AF-DX 116 was added to cells after labeling with [3H]inositol to give a final concentration of 4 μM. After a 10-min incubation (37°C and 5% CO₂), a small volume of 4-DAMP mustard was added (final concentration 40 nM), and the cells were allowed to incubate for 1 h at 37°C and 5% CO₂. Immediately before use, 0.4 mM 4-DAMP mustard was cyclized to the aziridinium ion by a 30-min incubation at 37°C in 10 mM NaKPO₄, pH 7.4 (Thomas and Ehlert, 1992). After 4-DAMP mustard treatment, cell monolayers were washed 3 times with DMEM to remove AF-DX 116 and unreacted 4-DAMP mustard and its transformation products.

**Preliminary Analysis of Agonist Concentration-Response Curves**

The maximal response (Eₘₐₓ), concentration of agonist eliciting a half-maximal response (EC₅₀), and the Hill slope (n) were estimated from the agonist concentration-response curve by nonlinear regression analysis using Prism (GraphPad Software, Inc., San Diego, CA). The data for agonist-mediated inhibition of cAMP accumulation were fitted to the following equation:

\[ R = \frac{P}{100} \left( 100 - \frac{X^n}{E_{\text{max}} + EC_{50}} \right) \]  

(1)

in which P denotes cAMP accumulation elicited by forskolin or isoproterenol in the absence of muscarinic agonist and Eₘₐₓ denotes the maximal percent inhibition of cAMP accumulation. The agonist concentration-response curve for stimulation of phosphoinositide hydrolysis and enhancement of forskolin-stimulated cAMP accumulation in pertussis toxin treated cells was fitted to the following equation:

\[ R = \frac{X^nE_{\text{max}}}{EC_{50} + X^n + P} \]  

(2)

In this equation, P denotes the basal value of phosphoinositide hydrolysis measured in the absence of agonist. The dissociation constant of an antagonist (Kᵦ) was estimated on the basis of its ability to antagonize the response to an agonist competitively. The following equation was used to estimate Kᵦ:

\[ Kᵦ = \frac{I}{CR - 1} \]  

(3)

In this equation, I denotes the concentration of the antagonist used in the assay, and CR denotes the EC₅₀ value of the agonist measured in the presence of the antagonist divided by that measured in its absence.

**Estimation of RAᵦ**

To reduce error in the estimation of RAᵦ, we always tested the standard agonist carbachol together with the test agonist in the same experiment. In most instances, all five agonists were tested simultaneously. Our methods for the estimation of RAᵦ, represent a further refinement in our previously published methods (Ehlert et al., 1999; Ehlert and Griffin, 2001). We define the RAᵦ value of test agonist B relative to standard agonist A as the product of the observed intrinsic efficacy and affinity of agonist B divided by that for agonist A:

\[ RAᵦ = \frac{\epsilon_B(1/K_A)}{\epsilon_A(1/K_B)} \]  

(4)

In this equation, εₐ and εₖ denote the observed intrinsic efficacies, and Kᵦ and Kᵦ denote the observed dissociation constants of agonists A and B, respectively. In the first step of this analysis, the concentration-response curves of the test agonist B and the standard agonist A are analyzed by nonlinear regression analysis according to eqs. 1 or 2 to estimate the EC₅₀, Eₘₐₓ, and Hill slope values. If the Eₘₐₓ values of the two agonists are the same, then the RAᵦ value of agonist B expressed relative to A can be calculated as (Ehlert et al., 1999):

\[ RAᵦ = \frac{EC_{50,A}}{EC_{50,B}} \]  

(5)

In this equation, subscripts are used to denote the EC₅₀ values of A and B. When the Eₘₐₓ value of the test agonist is different from that of the standard agonist, the analysis is more complicated, and three different methods have been developed to estimate RAᵦ, in this situation, each with its own advantages and disadvantages. These are 1) a null method, which is independent of the relationship between occupancy and response, 2) a method based on the operational model, assuming a logistic relationship between occupancy and response, and 3) a special case of the operational model in which the Hill slope of the agonist concentration-response curve is equivalent to 1.

**Null Method.** The null method involves comparing equiactive concentrations of the test agonist B and the standard agonist A. In our description of the method, we designate the standard agonist as the agonist with the larger Eₘₐₓ. An advantage of this method is that it is suitable for the analysis of any shape of concentration-response curve as long as the curve is a continuous, increasing function of the agonist concentration. The method involves three steps: 1) estimation of pairs of equiactive concentrations of agonist A and B, 2) nonlinear regression analysis of these equiactive concentrations according to eq. 8, and 3) calculation of the RAᵦ value using eq. 9, which requires parameter estimates from step 2.

Pairs of equiactive agonist concentrations (Aᵢ and Bᵢ) are estimated as follows. The values of Bᵢ are simply those of the concentration-response curve of test agonist B, with the subscript i denoting the different concentrations. Each corresponding Aᵢ value is estimated from the concentration-response curve of agonist A by interpolating an Aᵢ value that yields a response equivalent to that elicited by Bᵢ. This interpolation is shown graphically in Fig. 3. Different methods of interpolation can be used, depending upon the shape of the concentration-response curve of A. In our experiments, the responses to all of the agonists were consistent with eqs. 1 and 2. Consequently, we rearranged these equations to solve for the agonist concentration (X) as a function of the response (R). Thus, eq. 1 was rearranged into the following form to interpolate Aᵢ values from the concentration-response curve for inhibition of cAMP accumulation:

\[ Aᵢ = EC_{50,A} \left( \frac{1}{100 \left( \frac{EC_{\text{max},A}}{R_{i-B}} - 1 \right)} \right)^{1/\epsilon_A} \]  

(6)

In this equation, Rᵢ-B denotes the response elicited by the i th concentration of B (Bᵢ), and EC₅₀-A, Eₘₐₓ-A, and εᵦ denote the best-fitting parameter estimates of the concentration-response curve of the standard agonist A. For agonist-stimulated phosphoinositide hydrolysis in HEK293 cells and agonist-mediated enhancement of forskolin-stimulated cAMP accumulation in pertussis toxin-treated CHO M₂ cells, eq. 2 was rearranged to the following to enable an interpolation of Aᵢ values:

\[ Aᵢ = EC_{50,A} \left( \frac{1}{R_{i-B} - 1} \right)^{1/\epsilon_A} \]  

(7)

Thus, by substituting in the response values of the concentration-response curve to B into the corresponding eqs. 6 and 7, it was possible to estimate a set of Aᵢ values equiactive to those of Bᵢ. The resulting pairs of equiactive agonist concentrations were fitted to the following equation by nonlinear regression analysis:

\[ \log(Aᵢ) = \log \left( \frac{BqKₐ}{B(1 - q) + pKₐ} \right) \]  

(8)
The derivation of this equation is given under Appendix, and it represents the log form of eq. 19. The parameters p and q are defined under Appendix (see eqs. 20 and 21) and are equivalent to the observed affinity and efficacy of B relative to A, respectively. As described under Results, there are an infinite number of parameter estimates that yield the least-squares fit; however, the solution set is unique in that the ratio q/p is constant. Thus, it was possible to estimate q/p by fixing the parameter $K_B$ to an arbitrarily high value and determining the values of q and p that minimized the residual sum of squares. Additional details of the regression analysis are given under Results, and a method for obtaining initial parameter estimates for the iterative procedure is given in the last section under Appendix. The $R_A$ value can be calculated as

$$\text{RA}_i = \frac{q}{p}$$  \hspace{1cm} (9)

Substitution of eqs. 20 and 21 for p and q shows that eq. 9 reduces to eq. 4. It is possible to estimate the dissociation constant of the test agonist ($K_A$) as

$$K_B = pK_A$$  \hspace{1cm} (10)

in which $K_A$ denotes the constant to which $K_A$ was fixed during regression analysis.

**Operational Model.** When the agonist concentration-response curve is consistent with a logistic equation (eq. 1 or 2), it is possible to estimate the $R_A$ value of a partial agonist B relative to a more efficacious agonist A by first fitting the concentration-response curves of A and B to the operational model of Black and Leff (1983) by nonlinear regression analysis:

$$R = \frac{X^m M_{sys}}{X^m + (X + K)^m}$$  \hspace{1cm} (11)

in which R denotes the response to the agonist, X denotes the concentration of A or B, and $\tau$ is defined in eq. 25 under Appendix. For the experiments involving agonist stimulation of phosphoinositide hydrolysis and stimulation of cAMP accumulation in CHO M2 cells treated with pertussis toxin, the response denotes the measurement minus the basal value observed in the absence of agonist. For the experiments involving agonist-mediated inhibition of forskolin- or isoproterenol-stimulated cAMP accumulation, the response represents the percentage inhibition of cAMP accumulation. Equation 11 is essentially equivalent to eq. 24 under Appendix. The subscript j is used to denote different agonists. Thus, $K_j$ denotes the dissociation constants of A and B ($K_j$ and $K_A$), whereas $\tau_j$ denotes the $\tau$ values of A and B ($\tau_A$ and $\tau_B$), respectively. The concentration-response curves to the standard agonist A and the test agonist B are analyzed simultaneously by global nonlinear regression analysis, sharing the estimates of $M_{sys}$ and $\tau$ between the curves and allowing for individual estimation of $\tau$ and $K_j$ for the standard agonist ($\tau_A$ and $K_A$) and the test agonist ($\tau_B$ and $K_B$). If the standard agonist is a full agonist with a receptor reserve, then an infinite number of parameter estimates will yield a least-squares fit as described under Results. However, the solution set does yield the best estimates of $m$, $M_{sys}$, $\tau$, $K_B$, and the ratio $\tau_A/K_A$. Thus, it may be necessary to fix $K_A$ to an arbitrarily high value and determine the values of the other parameters that yield a least-squares fit as described under Results. Additional details of the regression analysis are given under Results, and a method for obtaining initial parameter estimates for the iterative procedure is given in the last section under Appendix. The $R_A$ value of B relative to A is calculated as

$$\text{RA}_i = \frac{\tau_A/K_B}{\tau_A/K_A} + \frac{\tau_B/K_A}{\tau_B/K_B}$$  \hspace{1cm} (12)

By making the appropriate substitutions for $\tau$ (eq. 25 under Appendix), it can be shown that eq. 12 reduces to eq. 4.

**Hill Slope Equals 1.** When the Hill slope of the agonist concentration-response curve is equal to 1, the $R_A$ value of agonist B relative to standard agonist A is calculated as

$$R_A = \frac{E_{max-B} EC_{50-A}}{E_{max-A} EC_{50-B}}$$  \hspace{1cm} (13)

If the definition of $\tau$ (eq. 25) is substituted into eqs. 27 and 28, and these latter equations are then substituted for the $E_{max}$ and $EC_{50}$ values into eq. 13, then it can be shown that eq. 13 reduces to eq. 4.

**Drug and Chemicals.** Drugs and chemicals were obtained from the following sources: [3H]adenine and [3H]inositol, PerkinElmer Life and Analytical Sciences (Boston, MA); DMEM and trypsin-EDTA (In Vitrogen, Carlsbad, CA); carbachol, McN-A-343, and oxtremorine-M (Sigma-Aldrich, St. Louis, MO); and AF-DX 116 (Boehringer Ingelheim Pharmaceutical, Ridgefield, CT). 4-DAMP mustard was synthesized as described previously (Thomas et al., 1992). The enantiomers of aceclidine were synthesized and resolved as described by Ringdahl et al. (1979).

**Results.**

**Phosphoinositide Hydrolysis.** We investigated the ability of muscarinic agonists to elicit phosphoinositide hydrolysis through the $M_4$ muscarinic receptor in HEK G15 cells. The highly efficacious muscarinic agonist, oxtremorine-M, elicited a small phosphoinositide response in HEK G15 cells, presumably through activation of an endogenous muscarinic receptor. When the latter cells were transiently transfected with the human $M_4$ muscarinic receptor, the resulting cells (HEK G15 $M_4$) exhibited a robust phosphoinositide response to oxtremorine-M (Fig. 1a). The response was resistant to treatment with pertussis toxin (0.1 μg/ml, overnight incubation; data not shown). To identify the endogenous receptor mediating phosphoinositide hydrolysis in HEK G15 cells, we characterized the antagonism of the response by subtype-selective muscarinic antagonists. When measured in the presence of either AF-DX 116 (10 μM) or pirenzepine (1.58 μM), the concentration-response curve to oxtremorine-M shifted to the right 17- and 14-fold, respectively. These antagonist-induced shifts correspond to calculated $pK_I$ values of 6.20 ± 0.095 and 6.89 ± 0.19 for AF-DX 116 and pirenzepine, respectively (see eq. 3). Collectively, these $pK_I$ values agree with the corresponding binding affinities ($pK_D$ values) of AF-DX 116 and pirenzepine at the human $M_4$ muscarinic receptor (6.10 ± 0.06 and 6.59 ± 0.03) but not those of the $M_1$ (6.24 ± 0.03 and 7.77 ± 0.03), $M_2$ (7.27 ± 0.05 and 5.96 ± 0.05), $M_3$ (6.96 ± 0.12 and 7.23 ± 0.02), or $M_5$ muscarinic receptor (5.29 ± 0.11 and 6.55 ± 0.06). Consequently, we conclude that the $M_4$ subtype mediates phosphoinositide hydrolysis in HEK G15 cells.

To eliminate this $M_4$ response, we treated cells with the irreversible muscarinic antagonist 4-DAMP mustard in combination with the competitive $M_4$ selective antagonist AF-DX 116 for 60 min followed by washing. This treatment has been shown to cause 93% inactivation of $M_4$ receptors while only inactivating $M_2$ by 22% (Griffin et al., 2003). As shown in Fig. 1b, 4-DAMP mustard treatment completely eliminated the response to the endogenous $M_4$ receptor in HEK G15 cells while having little inhibitory effect on that measured in HEK G15 $M_4$ cells.

Having developed a simple method for investigating the
signaling of the M2 receptor through G\textsubscript{i15}, we measured the ability of a group of agonists with varying potencies and intrinsic efficacies to stimulate phosphoinositide hydrolysis in HEK G\textsubscript{i15} M2 cells, which had been previously treated with 4-DAMP mustard. We also treated these cells with pertussis toxin (0.1 μg/ml) in combination with AF-DX 116 (4 μM) for 1 h and washed three times. The data represent the mean values ± S.E.M. of six experiments each done in triplicate.

Estimation of Agonist RA Values for Phosphoinositide Hydrolysis in HEK Cells. We estimated the RA\textsubscript{A} values of agonists from the data shown in Fig. 2a using the procedures described under Materials and Methods. Figure 3 illustrates our use of the null method to estimate the RA\textsubscript{A} value of McN-A-343 relative to carbachol. First, predicted concentrations of carbachol that elicit responses equivalent to those of the concentration-response curve to McN-A-343 were interpolated as shown in Fig. 3a using eq. 7. These interpolated concentrations are plotted against the corresponding equieffective concentrations of McN-A-343 in Fig. 3b. Equation 8 was fitted to the data by nonlinear regression analysis to estimate p, q, and K\textsubscript{A}; however, an infinite number of parameter values yield the same least-squares fit. Consequently, it is impossible to find a solution set consisting of single values of these parameters. When the value of K\textsubscript{A} was fixed as a constant; however, it was possible to obtain a least-squares fit corresponding to unique values of p and q and the constant K\textsubscript{A}. As the value of log K\textsubscript{A} was set at various values between −7.5 and an arbitrarily high value of −1, the best-fitting values of p and q decreased proportionately so that their ratio remained constant as well as the residual sum of squares (RSS). Thus, it was possible to estimate the ratio p/q even though it was impossible to estimate unique values of K\textsubscript{A}, p, and q. The RA\textsubscript{A} values of McN-A-343, expressed relative to carbachol is simply calculated as the ratio q/p (see eq. 9). The RA\textsubscript{A} values of S- and R-aceclidine were estimated using a similar strategy. Because the E\textsubscript{max} value of carbachol was less than those of oxotremorine-M and S-aceclidine, we first calculated the RA\textsubscript{A} values of carbachol and S-aceclidine relative to oxotremorine-M and then expressed the resulting RA\textsubscript{A} values relative to carbachol. A summary of these estimates is given in Table 1. We estimated the dissociation constants of the partial agonists R-aceclidine (pK\textsubscript{D} = 3.81 ± 0.37) and McN-A-343 (pK\textsubscript{D} = 4.63 ± 0.15) using eq. 10.

We also used the operational model to estimate the RA\textsubscript{A} values of the agonists. First, we fitted the concentration-response curves shown in Fig. 2a to eq. 11 using global nonlinear regression analysis, sharing the estimates of M\textsubscript{sys} and m among the curves and estimating unique values of τ and K for each curve. It was possible to obtain unique estimates of all of the parameters using this method; however, the estimates of τ and K for the full agonists were poorly defined and exhibited huge standard errors as well as the estimate of M\textsubscript{sys}. This limitation arises with full agonists or, in other words, whenever the E\textsubscript{max} value is approximately equal to the maximum response of the system (M\textsubscript{sys}). Nevertheless, the ratio of τ/K for each agonist was well defined. Thus, it was possible to estimate the RA\textsubscript{A} value of each agonist by taking the ratio of the τ/K of each agonist relative to that of carbachol as shown by eq. 12. The RA\textsubscript{A} estimate of each agonist is listed in Table 1 together with the values estimated by the null method. It can be seen that there is close agreement between the two methods. It was also possible to estimate the dissociation constants of the partial agonists, R-aceclidine (pK\textsubscript{D} = 3.64 ± 0.46) and McN-A-343 (pK\textsubscript{D} = 4.93 ± 0.12).

To illustrate the unique relationship among parameter estimates in the operational model, we fixed the log K\textsubscript{A} value of the standard agonist carbachol (K\textsubscript{carb}) at various values between −6 and −1 and used nonlinear regression analysis to estimate the best-fitting values of the other parameters. Figure 4b shows the results of this regression analysis for the estimation of the RA\textsubscript{A} value of McN-A-343 relative to carbachol. It can be seen that once the log K\textsubscript{carb} value is −2 to −5, the RSS drops to a minimum and remains at this low value no matter how large the increase in K\textsubscript{carb}. Over this range of K\textsubscript{carb} values, the estimates of m, M\textsubscript{sys}, τ\textsubscript{McN}, and K\textsubscript{McN} are constant. As log K\textsubscript{carb} increases from −5 to −1, there is a
proportional increase in $\tau_{\text{carb}}$, such that the ratio of $\tau_{\text{carb}}/K_{\text{carb}}$ is constant (Fig. 4c). Figure 4c illustrates that the ratio of $\tau_{\text{McN}}/K_{\text{McN}}$ is also constant over the domain $\log K_{\text{carb}} = -5$ as well as the estimate of $RA_i$. In summary, the results in Fig. 4 illustrate that it is possible to estimate the $RA_i$ value of a partial agonist relative to a full agonist by setting the $K_A$ value of the full agonist to an arbitrarily high value and using nonlinear regression analysis to estimate the other parameters in the operational model (eq. 11). The $RA_i$ value can then be calculated from these parameters using eq. 12. This analysis for the agonists in Fig. 2 also yielded estimates of $m$ ($0.721 \pm 0.044$), $M_{\text{sys}}$ (104 \pm 4.7), and the dissociation constants of the partial agonists as listed in the previous paragraph. Figure 4a shows the best fit of the operational model to the concentration-response curves of carbachol and McN-A-343 using this method.

We also estimated the $RA_i$ values of the agonists for stimulation of phosphoinositide hydrolysis in HEK $G_{a15}$ cells using the simple calculation given in eq. 13, which is valid provided that the Hill slopes differ from 1, and the $E_{\text{max}}$ values of the agonists are not less than 50\% of that of a full agonist. Table 1 lists these estimates. It can be seen that there is close agreement between the estimates using eq. 13 and those estimated with the null method and operational model for all of the agonists exhibiting similar $E_{\text{max}}$ values (i.e., oxotremorine-M, carbachol, S-acecclidine, and R-acceclidine). However, the simple estimate of the $RA_i$ for McN-A-343 is -3-fold greater than those estimated using the other two methods, which illustrates the flaw in using the simple method when the Hill slopes differ from 1 and there is a large difference in $E_{\text{max}}$ between the standard and test agonist.

We also estimated the $RA_i$ values of carbachol, oxotremorine-M, S-acceclidine, R-acceclidine, and McN-A-343 for their effects on phosphoinositide hydrolysis in HEK $G_{a15}$ cells that had not been transfected with the $M_2$ receptor, and these results are summarized in Table 2.

**Agonist-Mediated Inhibition of cAMP Accumulation in CHO $M_2$ Cells.** To determine whether the $RA_i$ values of agonist varied depending upon the $G$ protein with which the $M_2$ receptor interacts, we measured agonist-mediated inhibition of forskolin-stimulated cAMP accumulation in CHO cells stably transfected with the human $M_2$ muscarinic receptor (Fig. 5a). Carbachol, oxotremorine-M, and the enantiomers of aceclidine behaved as full agonists and caused a maximal inhibition of cAMP accumulation at high concentrations (i.e., 1.0 mM). Consequently, we shared the estimate of $E_{\text{max}}$ among the full agonists to prevent an overestimation of the $E_{\text{max}}$ of $R$-aceclidine. The $E_{\text{max}}$ values of the other full agonists are listed in Table 3. The $RA_i$ values of the $M_2$ receptor were calculated according to eq. 13 and are also listed in Table 3. Note that when the standard agonist and the test agonist exhibit the same $E_{\text{max}}$. eqs. 5 and 13 yield the same result. The $RA_i$ value of McN-
### TABLE 1
Activity of muscarinic agonists for stimulating phosphoinositide hydrolysis in HEK G_{15} M_{2} cells

The data are from Fig. 2a. The data represent the mean estimates ± S.E.M. The values in parentheses beside some of the estimates are the log means ± S.E.M.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>E_{max}^a</th>
<th>EC_{50}</th>
<th>Hill Slope</th>
<th>R_A_i</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>µM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxotremorine-M</td>
<td>103.3 ± 5.3</td>
<td>0.18 (−6.76 ± 0.051)</td>
<td>0.75 ± 0.021</td>
<td>7.1 (0.85 ± 0.065)</td>
</tr>
<tr>
<td>Carbachol</td>
<td>100</td>
<td>1.1 (−5.92 ± 0.041)</td>
<td>0.71 ± 0.071</td>
<td>1.0 (0.0)</td>
</tr>
<tr>
<td>S-Aceclidine</td>
<td>103.1 ± 5.8</td>
<td>1.79 (−5.75 ± 0.070)</td>
<td>0.99 ± 0.088</td>
<td>0.66 (−0.18 ± 0.066)</td>
</tr>
<tr>
<td>R-Aceclidine</td>
<td>90.9 ± 7.5</td>
<td>17 (−4.71 ± 0.11)</td>
<td>0.64 ± 0.10</td>
<td>0.052 (−1.28 ± 0.11)</td>
</tr>
<tr>
<td>McN-A-343</td>
<td>35.5 ± 8.3</td>
<td>5.3 (−5.28 ± 0.096)</td>
<td>1.22 ± 0.23</td>
<td>0.076 (−1.12 ± 0.086)</td>
</tr>
</tbody>
</table>

^a E_{max} is expressed relative to that for carbachol.

### TABLE 2
Agonist activity for stimulating phosphoinositide hydrolysis in HEK G_{15} cells

The data are from Fig. 2b. The data represent the mean estimates ± S.E.M. The values in parentheses beside some of the estimates are the log means ± S.E.M.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>E_{max}^a</th>
<th>EC_{50}</th>
<th>Hill Slope</th>
<th>R_A_i</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>µM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxotremorine-M</td>
<td>123 ± 8.6</td>
<td>2.6 (−5.59 ± 0.035)</td>
<td>0.87 ± 0.11</td>
<td>4.7 (0.67 ± 0.021)</td>
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<tr>
<td>Carbachol</td>
<td>100</td>
<td>9.3 (−5.03 ± 0.023)</td>
<td>0.99 ± 0.05</td>
<td>1.0 (0.0)</td>
</tr>
<tr>
<td>S-Aceclidine</td>
<td>55.4 ± 9.4</td>
<td>12.1 (−4.92 ± 0.22)</td>
<td>0.76 ± 0.08</td>
<td>0.42 (−0.38 ± 0.16)</td>
</tr>
<tr>
<td>R-Aceclidine</td>
<td>17.5 ± 2.0</td>
<td>81 (−4.09 ± 0.19)</td>
<td>1.0^a</td>
<td>0.021 (−1.68 ± 0.16)</td>
</tr>
<tr>
<td>McN-A-343</td>
<td>12.2 ± 6.7</td>
<td>38 (−4.42 ± 0.27)</td>
<td>1.0^a</td>
<td>0.020 (−1.70 ± 0.29)</td>
</tr>
</tbody>
</table>

^a The Hill slope was constrained to 1.
A-343 was estimated using eq. 13 as well as the null method and operational model, and these estimates are also shown in Table 3. In this instance, eq. 13 gave results similar to those for the other two methods, probably because the Hill slopes of carbachol and McN-A-343 were close to 1. The estimates of the negative logarithm of the dissociation constant of McN-A343 using the null method (pK = 4.16 ± 0.24) and the operational model (pK = 4.07 ± 0.16) were approximately the same.

Comparison of the data in Tables 1 and 3 shows that whereas the RAi values of oxotremorine-M, carbachol, S-aceclidine, and R-aceclidine are similar, the value for McN-A-343 is ~10-fold greater in HEK G15 M2 cells relative to that measured in CHO M2 cells. This increase in the RAi value of McN-A-343 was associated with an average 4.5-fold increase in observed affinity (decrease in dissociation constant), indicating a small increase in observed intrinsic efficacy (~2-fold) as well. To verify that this difference can be attributed to different G proteins and not to the cells in which the M2 receptor was expressed, we investigated M2 receptor-mediated inhibition of isoproterenol-stimulated cAMP accumulation in HEK T2 cells stably expressing the human M2 muscarinic receptor (HEK T M2). Figure 5b shows the results of these experiments using carbachol and McN-A-343 as agonists. The results of these experiments are summarized in...
Table 4 where it can be seen that the RA value of McN-A-343 is similar to that measured in CHO M2 cells but only $\sim 1/10$ that measured in HEK G_{a15} M2 cells. These results suggest that McN-A-343 preferentially directs signaling of the M2 receptor through G_{a15} relative to Gi.

**Agonist-Mediated Enhancement of Forskolin-Stimulated cAMP Accumulation.** Michal et al. (2001) have shown that the M2 receptor has biphasic effects on cAMP accumulation in CHO cells when forskolin is present. At low concentrations, muscarinic agonists cause a potent inhibition of cAMP accumulation, whereas at high concentrations, an enhancement of cAMP accumulation occurs. The inhibitory effect is mediated via Gi and is prevented by pertussis toxin treatment, whereas the stimulatory effect is mediated by Gs (Michal et al., 2001, 2007). After pertussis toxin treatment, only the less potent stimulatory phase is observed. Consequently we measured agonist concentration-response curves for stimulating cAMP accumulation in the presence of forskolin (10 μM) in CHO M2 cells that had been previously treated with pertussis toxin to prevent Gi-mediated inhibition of adenyl cyclase (Fig. 6a). Carbachol, oxotremorine-M, and S-aceclidine all exhibited a similar shaped sigmoid concentration-response curves with similar $E_{\text{max}}$ values, whereas R-aceclidine exhibited a bell-shaped curve with a lower maximum. McN-A-343 actually caused a concentration-dependent inhibition of cAMP accumulation. Because both McN-A-343 and R-aceclidine inhibited cAMP accumulation at high concentrations, we measured their effects on forskolin-stimulated cAMP accumulation in CHO cells that had not been transfected with the M2 muscarinic receptor.
Both agonists caused a concentration-dependent inhibition of forskolin-stimulated cAMP accumulation in untransfected CHO cells, suggesting that the compounds interfere with adenyl cyclase through a nonmuscarinic receptor mechanism at high concentrations. In contrast, carbachol had no such effect. Thus, in analyzing the concentration-response curve to $R$-aceclidine, we only used the data over the log molar concentration range of $10^{-5}$ to $10^{-3.5}$ $R$-aceclidine.

The estimates of the $RA_i$ values of carbachol, oxotremorine-M, $S$-aceclidine, and $R$-aceclidine for stimulation of cAMP accumulation were calculated using the three different methods, and these estimates are listed in Table 4. There is agreement among the three different estimates for a given agonist. Also the $RA_i$ values estimated for stimulation of cAMP accumulation agree generally with those estimated in the other assays.

**Analysis of the Loss of Activity of McN-A-343 in Stimulating cAMP Accumulation.** The lack of effect of McN-A-343 in enhancing forskolin-stimulated cAMP accumulation in CHO $M_2$ cells treated with pertussis toxin may indicate that the $RA_i$ value of McN-A-343 in this assay is less than that observed in the two other assays. Alternatively, it may be that this response is so insensitive that the relatively weak activity of McN-A-343 at the $M_2$ receptor simply does not register in this assay even though its $RA_i$ value may be similar to those observed in the other assays. To discriminate between these two possibilities we determined the loss in sensitivity to carbachol that occurs when stimulation of cAMP accumulation (CHO $M_2$ G$i$ assay) is measured instead of inhibition of cAMP accumulation (CHO $M_2$ G$i$ assay), and
determined whether an equivalent loss in sensitivity could eliminate the response to McN-A-343 in the CHO M2 Gs assay, assuming no change in its RA1 value. Figure 7a shows that the concentration-response curve of carbachol in the CHO M2 Gs assay is shifted to the right 26-fold compared with that for the CHO M2 Gi assay. From the perspective of the operational model, this loss in sensitivity can be attributed to a reduction in τ to a value only 3.9% of that measured in the CHO M2 Gi assay. If we apply the same loss in sensitivity to the concentration-response curve of McN-A-343 in the CHO M2 Gs assay, the resulting curve only reaches an Emax of 1.7% as shown in Fig. 8a by the theoretical curve for McN-A-343 in the CHO M2 Gi assay. This level of stimulation would have been impossible to detect, given the nonspecific inhibitory effect of McN-A-343 on cAMP accumulation. The apparent reduction in the τ value of carbachol between the two assays can be estimated in the same manner that one estimates RA1, except that in this instance, we are estimating the activity of carbachol in the CHO M2 Gs assay relative to its activity in the CHO M2 Gi assay. Because carbachol behaves as a full agonist in both assays, its τ value in the CHO M2 Gs assay relative to that of the CHO M2 Gi assay is simply equivalent to the ratio of its EC50 value in the Gi assay expressed relative to that for the Gs assay. We also carried out a similar type of analysis for the loss in sensitivity to carbachol in the CHO M2 Gs assay compared with the HEK Gs,15 M2 assay (Fig. 8b). In the latter case, the loss in sensitivity to carbachol corresponded to a reduction in τ of 77%, and this change nearly, but not completely, eliminated the response to McN-A-343, and it is impossible to tell from the data whether the actual RA1 value of McN-A-343 in the CHO M2 Gs assay is any less than those observed in the HEK Gs,15 M2 and CHO M2 Gi assays.

**Discussion**

We have described a method for estimating the product of observed affinity and intrinsic efficacy of an agonist for a receptor expressed relative to that of another using only a single concentration-response curve for each agonist. This measure (RA1) provides a means of converting EC50 and Emax values into a single estimate, enabling one to compare the activity of an agonist across different assays, regardless of whether the agonist behaves as a full or partial agonist. Our results show that carbachol, oxotremorine-M, and the enantiomers of aceclidine have similar activity across the different M2 assays, whereas McN-A-343 exhibits ~10-fold greater activity at the M2 receptor signaling through Gs,15 compared with Gi (Fig. 8a). This ability of McN-A-343 to select for M2-Gs,15 responses is important in drug screening because Gs,15 is often used to transduce signals through GPCRs.

If an agonist induces or selects a receptor conformation that preferentially interacts with Gi,15, then the agonist should exhibit a larger RA1 value for eliciting Gi,15 responses relative to a standard agonist that lacks this selectivity. Presumably, this phenomenon explains the selectivity of McN-A-343 for Gi,15 responses relative to Gi,15 responses. This selectivity for Gi,15 responses should be manifest when the agonist is assayed in different cells, each expressing a single G protein (e.g., Gi,15 or Gi). If both G proteins are expressed within the same cell and the receptor is in a dynamic equi-

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**Fig. 7.** Estimation of the theoretical concentration-response curve to McN-A-343 for enhancement of forskolin stimulated cAMP accumulation in pertussis toxin treated CHO M2 cells. The theoretical curves were generated assuming that the observed affinity and intrinsic efficacy of McN-A-343 were that same as that for inhibition of cAMP accumulation (a) or stimulation of phosphoinositide hydrolysis (b). a: The theoretical concentration-response curve to McN-A-343 for enhancement of cAMP accumulation was estimated, assuming that its reduced sensitivity for stimulating relative to inhibiting cAMP accumulation was the same as that observed for carbachol. b: The theoretical concentration-response curve to McN-A-343 for enhancement of cAMP accumulation was estimated, assuming that its reduced sensitivity for stimulating cAMP accumulation relative to stimulating inositol phosphate accumulation was the same as that observed for carbachol. Further details are given under Results. The experimental data for McN-A-343 and carbachol are from Figs. 2, 5, and 6.
librium with both, then the $RA_i$ value of an agonist for responses mediated through either G protein should be the same and equal to a weighted average of those observed in different cells (Leff et al., 1997). Bearing this hypothesis in mind, we treated HEK G$_{15}$ M$_2$ cells with pertussis toxin to prevent M$_2$ receptor signaling through G$_i$ when G$_{15}$ responses in HEK G$_{15}$ M$_2$ cells were measured so that our $RA_i$ estimate would reflect only the M$_2$-G$_{15}$ interaction. Nevertheless we also performed the same experiments without pertussis toxin treatment and observed essentially the same results for McN-A-343 and the other agonists (data not shown). Perhaps the selectivity of the McN-A-343-M$_2$ receptor complex for G$_{15}$ is so great that G$_i$ does not compete effectively and causes little perturbation of the M$_2$ receptor-G$_{15}$ interaction. Alternatively, these findings may suggest that different pools of M$_2$ receptors are dedicated to either G$_{15}$ or G$_i$ and that the M$_2$ receptor is not in equilibrium with both G proteins at the same time in HEK G$_{15}$ M$_2$ cells. We speculate that this compartmentalization might be maintained by scaffolding or accessory proteins as established for other systems (Ostrom et al., 2000).

It is possible that the M$_2$ receptor-mediated enhancement in forskolin-stimulated cAMP accumulation has little physiological relevance. We found that muscarinic agonists cause little or no increase in cAMP accumulation in pertussis toxin-treated CHO cells unless forskolin is present. Presumably, forskolin enhances the weak activation of G$_i$ by muscarinic agonists in CHO cells so that a substantial activation of adenylyl cyclase can be measured in cells treated with pertussis toxin. It has been shown that type VI adenylyl cyclase is abundantly expressed in CHO cells (Varga et al., 1998) and that this isozyme exhibits synergistic interactions between forskolin and G$_i$ (Sutkowski et al., 1994). Thus, although the M$_2$ receptor-G$_i$ interaction may be too weak to subserve a physiological role, it nonetheless provides an interesting opportunity to test whether the nature of the G protein with which the receptor interacts influences agonist activity.

The $RA_i$ values that we reported for muscarinic agonists in the CHO M$_2$ G$_i$ assay are consistent with the activity of these agonists reported in the electrically driven guinea pig left atrium (Fig. 8b). This preparation is a standard assay for the M$_2$ muscarinic receptor signaling through a G$_i$ mechanism. We estimated the $RA_i$ values of the agonists in this preparation from published reports of the EC$_{50}$ and $E_{\text{max}}$ values (Eltze et al., 1993; Barocelli et al., 2000) using eq. 13 and also using regression analysis (eq. 11) of the concentration-response curves for carbachol and McN-A-343 published by Christopolous and Mitchelson, 1997). The good agreement between the two sets of data illustrate the usefulness of the $RA_i$ estimate in predicting agonist activity at the same receptor-G protein signaling mechanisms across different assays.

One way to appreciate the significance of $RA_i$ is to consider a series of agonists in a hypothetical, highly sensitive assay in which the agonists would behave as full agonists, even those with little intrinsic efficacy. In this situation, the $RA_i$ values would be equivalent to the ratio of EC$_{50}$ values of the agonists expressed relative to a standard agonist. To illustrate this point, we show a comparison of the $RA_i$ values of muscarinic agonists for stimulating contractions in the guinea pig ileum with those for stimulating phosphoinositide hydrolysis in CHO cells transfected with the human M$_3$.

Fig. 8. Comparison of the $RA_i$ values of muscarinic agonists for eliciting different responses through M$_1$ (a and b) and M$_3$ (c) muscarinic receptors. a, the $RA_i$ values of muscarinic agonists for eliciting responses through the M$_2$ receptor coupled to G$_{15}$, G$_i$, and G$_s$ are shown. The data are from Tables 1, 3, and 5 and represent the mean values calculated using the null method and the operational model, except for the values of the full agonists in Table 3, which were calculated using eq. 13. b, the $RA_i$ values of muscarinic agonists for inhibiting cAMP accumulation in CHO M$_2$ cells and for inhibiting the electrically driven guinea pig left atrium are shown. The values for carbachol, the enantiomers of aceclidine and McN-A-343 were calculated using eq. 13 and the EC$_{50}$ and $E_{\text{max}}$ values published by Eltze et al. (1993) for the isolated atrium. Another estimate for McN-A-343 in the atrium was calculated from the data of Christopolous and Mitchelson (1997) by regression analysis using eq. 11, and this estimate was averaged with that from Eltze et al. (1993). The $RA_i$ value for oxotremorine-M was estimated from the data of Barocelli et al. (2000) and was first calculated relative to oxotremorine using eq. 13 and then expressed relative to carbachol using the data of Eltze et al. (1993). c, the $RA_i$ values of muscarinic agonists for eliciting phosphoinositide hydrolysis in CHO M$_3$ cells and contraction of the guinea pig ileum are shown. The latter data are from Ehlert et al. (1999).
TABLE 5
Agonist activity for enhancing forskolin-stimulated cAMP accumulation in pertussis toxin-treated CHO M2 cells
The data are from Fig. 2a. The data represent the mean estimates ± S.E.M. The values in parentheses beside some of the estimates are the log means ± S.E.M.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>$E_{\text{max}}$</th>
<th>EC$_{50}$</th>
<th>Hill Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxotremorin-M</td>
<td>93.9 ± 5.4</td>
<td>1.35 (−5.87 ± 0.057)</td>
<td>1.8 ± 0.23</td>
</tr>
<tr>
<td>Carbachol</td>
<td>100</td>
<td>5.01 (−5.30 ± 0.068)</td>
<td>1.12 ± 0.14</td>
</tr>
<tr>
<td>S-Accideline</td>
<td>93.2 ± 3.6</td>
<td>18.0 (−4.75 ± 0.12)</td>
<td>1.38 ± 0.11</td>
</tr>
<tr>
<td>R-Accideline</td>
<td>32.0 ± 2.1</td>
<td>19.6 (−4.71 ± 0.14)</td>
<td>2.2 ± 0.18</td>
</tr>
</tbody>
</table>

RA$_i$ values are related to the initial slope of the agonist concentration-response curve. By taking the first derivative (dy/dX) of the operational model (eq. 11) with respect to the agonist concentration (X) and evaluating its limit as the agonist concentration approaches 0, it is possible to estimate the initial slope of an agonist concentration-response curve. If the initial slope for agonist B is divided by that of a standard agonist A, the result is RA$_i^m$, where m denotes the transducer slope factor in the operational model:

$$\lim_{X \to 0} \frac{dy}{dX} = \frac{\tau_B(1/K_B)}{\tau_A(1/K_A)} = RA_i^m$$  \hspace{1cm} (14)

In these equations, X denotes the concentration of agonist with subscripts for test agonist (B) and standard agonist (A). Thus, when considered from this perspective, the RA$_i^m$ is a relative measure of the sensitivity of receptor-G protein complexes for different agonists. The initial slope of the plot of output against signal intensity is a common means of expressing the sensitivity of a transducer in electronics and engineering.

GPCRs are unique in that observed affinity is influenced by the concentration of guanine nucleotide in the cytosol, particularly for $G_i$ and $G_s$ linked receptors. As the concentration of GTP and GDP increases, observed affinity decreases. This change in affinity occurs with a corresponding opposite change in intrinsic efficacy such that the product of observed affinity and intrinsic efficacy remains constant (Ehlert and Rathbun, 1990; Ehlert, 2000). Consequently the RA$_i$ estimate is unperturbed by changes in the guanine nucleotide concentration, making it an ideal measurement of activity in cells with different concentrations of GTP or in broken cell assays in which the guanine nucleotide concentration is manipulated [e.g., agonist stimulated guanosine 5’-O-(3-[[35S]thiotriphosphate binding].

Although the regression methods that we used for estimating RA$_i$ values used the null method (eq. 8) and the operational model (estimation of EC$_{50}$ and $E_{\text{max}}$) may seem complicated, a computer algorithm can be written to make these calculations automatic, like the estimation of EC$_{50}$ and $E_{\text{max}}$. We think that the RA$_i$ estimate has widespread application in comparing agonist activity across different assay systems and in detecting agonist-directed signaling. Analogous types of calculations can be used to analyze allosterism (Ehlert, 2005), site-directed mutagenesis (Ehlert, 2000) and the loss of function in knockout mice when more than one receptor contributes to the function (Tran et al., 2006).

**APPENDIX**

**Theoretical Basis for the Estimation of RA$_i$**

This section describes the theoretical basis for the equations used to estimate the RA$_i$ values of agonists. Our method for the estimation of RA$_i$ is based on a further development of our previous methods (Ehlert et al., 1999; Ehlert and Griffin, 2001). In our analysis we assume that the amount of receptor activation to agonist X is proportional to the stimulus as defined by Furchgott (1966):

$$S = \frac{X e^R_T}{X + K_X}$$ \hspace{1cm} (16)

In these equations, $e_X$ denotes the intrinsic efficacy of X, $R_T$ denotes the concentration of functional receptors, and $K_X$ denotes the equilibrium constant. The response to agonist X can be represented as a function (f) of the stimulus:

$$\text{response} = f\left(\frac{X e^R_T}{X + K_X}\right)$$ \hspace{1cm} (17)

Below, three different approaches for estimating the RA$_i$ value of an agonist relative to a standard agonist are described. The first involves eliminating the stimulus-response function from the analysis through the use of a null method. The second involves the use of the operational model to describe the stimulus-response function, and the third involves a special case of the operational model in which the Hill slope of the concentration-response curve equals 1.

**Null Method.** One approach to estimation of RA$_i$ is to compare equivalent tissue responses to the two agonists so that the unknown relationship between the stimulus and response (f) is eliminated. By using this approach, the relationship describing equivalent tissue responses to agonist B and standard agonist A is given by

$$f\left(\frac{A_i e_A R_T}{A_i + K_A}\right) = f\left(\frac{B_i e_B R_T}{B_i + K_B}\right)$$ \hspace{1cm} (18)
in which $A_i$ and $B_i$ denote the equiactive concentrations of agonists $A$ and $B$, respectively. This equation simplifies to

$$A_i = \frac{B_i q K_A}{B_i (1 - q) + p K_A}$$

in which

$$p = \frac{K_B}{K_A}$$

$$q = \frac{\epsilon_B}{\epsilon_A}$$

As described under Materials and Methods (eq. 13), the $RA_i$ value can be estimated from the ratio $q/p$.

**Operational Model.** Although the null method just described has the advantage of being applicable for any type of stimulus-response function (i.e., any shape of concentration-response curve), it is widely observed that agonists usually exhibit logistic concentration-response curves. Indeed, a curve-fitting procedure based on the following logistic equation is the most common computational method that investigators use to estimate the $E_{\text{max}}$ and $EC_{50}$:

$$\text{response} = \frac{X^p E_{\text{max}}}{X^p + EC_{50}^{-q}}$$

in which $n$ denotes the Hill slope. Several investigators have shown that if the input to the stimulus-response function ($f$ is the stimulus (i.e., product of receptor occupancy and intrinsic efficacy) and the output obeys the logistic function just described, then the stimulus-response function must be the following (Furchgott, 1966; Mackay, 1981; Kenakin and Beek, 1982; Black and Leff, 1983):

$$\text{response} = \frac{S^m M_{\text{sys}}}{S^m + K_E^m}$$

in which $M_{\text{sys}}$ denotes the maximum response of the system, $K_E$ denotes the sensitivity of the stimulus-response function, and $m$ denotes the transducer slope factor. This exponent is related to, but not identical to, the Hill slope of the agonist concentration-response curve. Substitution of eq. 16 for $S$ in eq. 23 above followed by simplification yields

$$\text{response} = \frac{X^m M_{\text{sys}}}{X^m + (X + K_E)^m}$$

in which

$$\tau_X = \frac{\epsilon_X R_T}{K_E}$$

As described under Materials and Methods, the $RA_i$ value of an agonist can be estimated from the $K_X$ and $\tau_X$ values of the agonist (see eq. 12).

In situations in which the transducer slope factor $m$ in eq. 24 equals 1, the Hill slope of eq. 22 is also equal to 1 ($n = 1$), and eq. 24 reduces to

$$\text{response} = \frac{X F_{\text{max}}}{X + EC_{50}}$$

in which

$$E_{\text{max}} = \frac{\tau_M M_{\text{sys}}}{\tau_X + 1}$$

$$EC_{50} = \frac{K_E}{\tau_X + 1}$$

If eq. 25 is substituted into eqs. 27 and 28 for $\tau_X$ and then the resulting equations are substituted in for the $E_{\text{max}}$ and $EC_{50}$ values in eq. 13 under Materials and Methods, it can be shown that eq. 13 equals eq. 4. Thus, if the Hill slopes are equal to 1, it is possible to estimate the $RA_i$ value of agonists from the $E_{\text{max}}$ and $EC_{50}$ values.

**Initial Parameter Estimates for Nonlinear Regression Analysis.** We used the Solver function in Excel (Microsoft Office, 2004) to obtain the least-squares fit to eqs. 8 and 11 when $RA_i$ was estimated by the null method and operational model, respectively. The approach is analogous to that described by Christopoulos and Mitchelson (1998), but the regression equations are changed appropriately for our analysis. This regression analysis requires initial parameter estimates, and below we describe a means to calculate these when analyzing a partial agonist ($B$) with a standard full agonist ($A$). For the null method, the value of $K_A$ is fixed to an arbitrarily high value (e.g., log molar $K_A = -1$) and the initial estimates of $p$ and $q$ ($p'$ and $q'$, respectively) are estimated as follows:

$$p' = \frac{EC_{50-B}}{K_A}$$

$$q' = \frac{E_{\text{max-B}} EC_{50-A}}{E_{\text{max-A}} K_A}$$

In eqs. 29 and 30, $K_A$ denotes the arbitrarily high value to which $K_A$ is set as a constant for the regression analysis. For the operational model, the following initial parameter estimates for $\tau_A$, $\tau_B$, $K_B$, $m$, and $M_{\text{sys}}$ ($\tau_{A}'$, $\tau_{B}'$, $K_{B}'$, $m'$ and $M_{\text{sys}}'$, respectively) are used in situations in which the standard agonist was a full agonist:

$$\tau_{A}' = \frac{K_A}{EC_{50-A}}$$

$$\tau_{B}' = \frac{E_{\text{max-B}}}{E_{\text{max-A}}}$$

$$K_{B}' = EC_{50-B}$$

$$m = 1$$

$$M_{\text{sys}}' = E_{\text{max-A}}$$

In eq. 31, $K_A$ denotes the arbitrarily high value to which $K_A$ is set as a constant for the regression analysis.

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**References**


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