The Impact of Orthosteric Radioligand Depletion on the Quantification of Allosteric Modulator Interactions

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ABSTRACT
Radioligand binding assays remain a common method for quantifying the effects of allosteric modulators at G protein-coupled receptors. The allosteric ternary complex model (ATCM) is the simplest model applied to derive estimates of modulator affinity ($K_B$) and cooperativity ($n_c$), which are necessary for understanding structure-activity relationships. However, the increasing drive toward assay miniaturization in modern drug discovery may lead to conditions where appreciable ligand depletion occurs in the assay. Theoretical simulations investigating the impact of orthosteric radioligand depletion on the estimation of ATCM parameters revealed the following. 1) For allosteric inhibitors, application of the standard ATCM to data obtained under depletion conditions leads to an underestimation of $K_B$ and an overestimation of $n_c$. 2) For allosteric enhancers, the opposite was noted, but not always; the nonlinear regression algorithm is more likely to struggle to converge to a satisfactory solution of (nondepletion) ATCM parameters in this situation. 3) Application of a novel ATCM that explicitly incorporates orthosteric ligand depletion will yield more reliable model estimates, provided the degree of depletion is not high (<~50%). Subsequent experiments investigated the interaction between [3H]N-methyl-scopolamine and the allosteric enhancer, alcuronium, or inhibitor, gallamine, in the presence of increasing concentrations of $M_2$ muscarinic acetylcholine receptor and showed that application of an ATCM that explicitly incorporates radioligand depletion can indeed give more robust estimates of modulator affinity and cooperativity estimates than the standard model. These results have important implications for the quantification of allosteric modulator actions in binding-based discovery assays.

The major goal of drug discovery is the identification of chemical entities that specifically and effectively perturb target proteins to achieve a desired therapeutic action. G protein-coupled receptors (GPCRs) are one of the major protein families targeted for such purposes. An issue that is of direct relevance to the quantitation of GPCR-based assays is the phenomenon of ligand depletion, a condition in which the free ligand concentration in the system drops significantly (e.g., >10%) relative to the total concentration as a consequence of extensive removal by the binding process. This is seen when high-affinity ligands are used as probes to interrogate the activity of the target protein because in this situation, the receptor concentration (usually kept as low as possible) can nonetheless approach or even exceed the equilibrium dissociation constant of the probe; mass action would then ensure significant ligand depletion. Thus, the condition will probably arise in high-throughput assays if decreasing the volume of assay components for miniaturization leads to a decrease in the total amount of ligand added while retaining the same ligand concentration and total amount of receptors that are necessary to ensure a good signal/noise ratio.

Almost all equations routinely used to quantify ligand binding parameters assume that the free ligand concentration in the assay system has not changed significantly. However, this criterion is breached under ligand depletion conditions, and using the standard equations to derive the mechanistic parameters will yield incorrect estimates. This issue has been addressed before with orthosteric ligand binding studies, and equations to estimate orthosteric ligand binding parameters under depletion conditions have been derived and used successfully (Wells et al., 1980; Swillens, 1995). However, radioligand binding is also an important assay for validating the mechanism of action of novel allosteric ligands, as well as for facilitating the derivation of quantitative allosteric model parameters to assist structure-activity studies (Christopoulos and Kenakin, 2002). Yet, to our
knowledge, the consequences of ligand depletion on allosteric modulator screening and characterization have not been documented to date. The present study thus investigated the impact of ligand depletion on the measurement of allosteric GPCR-ligand interactions using simulations and experimental validation. A version of a simple allosteric ternary complex model (ATCM; Ehrlert, 1988; May et al., 2007) that allows for radioligand depletion was derived and applied to membrane-based radioligand binding assays using M2 muscarinic acetylcholine receptors (mACHRs). The results show that depletion of orthosteric radioligand significantly alters the allosteric ligand binding parameters obtained when fitting data using an ATCM that does not take radioligand depletion into consideration. The situation is more likely to arise with allosteric enhancers because they increase the affinity of the orthosteric probe and thus predispose the system to potential ligand depletion.

Materials and Methods

Materials. [3H]N-methyl-scopolamine methyl chloride ([3H]NMS; specific activity, 83 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). Dulbecco’s modified Eagle’s medium and hygromycin B were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum was purchased from ThermoTrace (Melbourne, Victoria, Australia). Alcuronium chloride was a generous gift from F. Hoffman-La Roche (Basel, Switzerland). All other materials were purchased from Sigma-Aldrich (St. Louis, MO).

Cell Membrane Preparation. Membrane preparations of CHO FlpIn cells stably expressing the M2 mACHR were done as described previously (Avlani et al., 2004, 2007). In brief, cells were grown and maintained in Dulbecco’s modified Eagle’s medium containing 20 mM HEPES, 10% fetal bovine serum, and 200 μg/ml hygromycin B and were grown for 4 days at 37°C in a humidified incubator containing 5% CO2:95% O2. Cells were then harvested by trypsinization followed by centrifugation (300g, 3 min), resuspension of the pellet in homogenization buffer (5 mM HEPES, 2.5 mM MgCl2, 2 mM EGTA), and resuspension of the pellet in HEPES buffer (110 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 1 mM MgSO4, 25 mM glucose, 50 mM HEPES, 58 mM sucrose, pH 7.4), repeated twice. The final pellet resuspended in 5 ml of ice-cold homogenization buffer (50 mM HEPES, 2.5 mM MgCl2, 2 mM EGTA) and then homogenized using a Polytron homogenizer for three 10-s intervals at maximum setting with 30-s cooling periods employed between each burst. The homogenate was centrifuged (1000g, 10 min, 25°C), the pellet was discarded, and the supernatant was recentrifuged at 30,000g for 30 min at 4°C. The resulting pellet was resuspended in 5 ml of HEPES buffer, and protein content was determined using the method of Bradford (1976). The homogenate was then aliquoted into 1-ml amounts and either used immediately or stored frozen at −80°C until required for radioligand binding assays.

Saturation Binding Assays. Three different amounts (10, 50, and 100 μg) of M2 CHO FlpIn cell membranes were incubated in 500-μl total volume of HEPES buffer containing concentrations of the orthosteric antagonist probe, [3H]NMS, ranging from 0.02 to 5 nM, for 60 min at 37°C. Non specific binding was defined using 10 μM atropine, Incubation was terminated by rapid filtration through Whatman GF/C filters using a Brandel cell harvester (Brandel Inc., Gaithersburg, MD). Filters were washed three times with 3-ml aliquots of ice-cold 0.9% NaCl and dried before the addition of 5 ml of scintillation cocktail (Ultima Gold; PerkinElmer Life and Analytical Sciences). Vials were then left to stand until the filters became uniformly translucent before radioactivity was determined using scintillation counting.

Equilibrium Binding Assays. Three different amounts (15, 75, and 150 μg) of M2 CHO FlpIn cell membranes were incubated in 500-μl total volume of HEPES buffer containing 0.2 nM [3H]NMS and a range of concentrations of the orthosteric agonist carbachol (100 nM–100 μM), orthosteric antagonist atropine (0.01 nM–10 μM), or the allosteric modulators alcuronium (3 nM–1 mM) or gallamine (100 nM–30 μM), at 37°C for 60 (orthosteric ligands) or 120 (allosteric modulators) min. Determination of nonspecific binding, termination of reaction, and determination of radioactivity were performed as described above.

Data Analysis. All of the data sets obtained in disintegrations per minute were converted to molar units. Nonspecific binding was subtracted from data sets when using the nondepletion version of ATCM. For the rest of the models, nonspecific binding was included as a fitted model parameter, whose value was found to increase with increasing ligand or membrane concentrations in all cases (data not shown).

All nonlinear regression was performed using Prism 4.03 (GraphPad Software Inc., San Diego, CA). Data sets of saturation binding assays were analyzed using the 1) nondepletion and 2) ligand depletion versions of the saturation binding equation (Motulsky and Christopoulos, 2004).

\[
Y = \frac{B_{\text{max}} \times [A]}{[A] + K_A} + \text{NS} \times [A]
\]

(1)

and

\[
Y = -b + \frac{\sqrt{b^2 - 4 \times a \times c}}{2 \times a}
\]

(2)

where \(a = -1 - \text{NS, } b = [A]T \times (2 \times \text{NS} + 1) + K_A(\text{NS} + 1) + B_{\text{max}}\) and \(c = -[A]T \times (K_A + [A]T) + B_{\text{max}}\).

\(B_{\text{max}}\) denotes the maximal density of binding sites, \(K_A\) is the radioligand equilibrium dissociation constant, NS is the fraction of nonspecific binding, \([A]T\) is the total amount of radioligand added, and \(Y\) represents the total amount of bound radioligand (specific binding + nonspecific binding). The quantity, \([A]\), represents the concentration of free orthosteric ligand as calculated by subtracting the total bound disintegrations per minute from the total added disintegrations per minute before conversion to molar units.

All data sets of equilibrium orthosteric ligand binding assays were analyzed using nonlinear regression (4) (Snedecor and Cochran, 1967) and ligand depletion (4) versions of a one-site competition binding equation (Swillens, 1995; Motulsky and Christopoulos, 2004).

\[
Y = \frac{B_{\text{max}} \times [A]}{[A] + K_A(1 + \frac{[I]}{K_I})} + \text{NS} \times [A]
\]

(3)

and

\[
Y = -b + \frac{\sqrt{b^2 - 4 \times a \times c}}{2 \times a}
\]

(4)

where \(a = -(\text{NS} + 1), b = (\text{NS} + 1) \times ([1 + 10^{\log K_I - \log K_L}] \times K_A + [A]T) + \text{NS} \times [A]T + B_{\text{max}}\) and \(c = -[A]T \times ([1 + 10^{\log K_I - \log K_L}] \times K_A + [A]T) \times \text{NS} + B_{\text{max}}\).

[I] is the concentration of competitive orthosteric ligand, and \(K_I\) is the competitive ligand equilibrium dissociation constant. The rest of the parameters are as described above.

Data sets of allosteric modulator equilibrium binding assays were analyzed using nonlinear regression (5) (Ehrlert, 1988; Lazareno and Birdsell, 1995; Christopoulos, 2000) and ligand depletion (7) versions of the ATCM.

\[
B_{\text{App}} = \frac{B_{\text{max}} \times [A]}{[A] + K_{\text{App}}} + \text{NS} \times [A]
\]

(5)

where

\[
K_{\text{App}} = K_A\left(\frac{1 + [B]K_B}{1 + (a \times [B])K_B}\right)
\]

(6)
and

$$B_{AB} = -\frac{b + \sqrt{b^2 - 4 \times a \times c}}{2 \times a}$$

where \(a = -1 - \text{NS}, b = [\alpha_{T}] \times (2 \times \text{NS} + 1) + K_{B},\)

and \(c = -[\alpha_{T}] \times (\text{NS} \times (K_{B} + [\alpha_{T}] + [R]).\)

\(B_{AB}\) represents the total amount of bound radioligand to the receptor, \([B]\) denotes the concentration and \(K_{B}\) is the equilibrium dissociation constant, respectively, for the allosteric modulator, and \(\alpha\) is the “cooperativity factor” for allosteric interaction between radioligand and modulator; values of \(\alpha > 1\) denote allosteric enhancement of binding affinity, whereas \(0 < \alpha < 1\) denotes allosteric inhibition of binding affinity. \([A]\) and \(K_{A}\) are as defined above. In this analysis, it is assumed that the allosteric modulator is itself not subject to appreciable depletion.

All affinity and cooperativity parameters were estimated as logarithms (Christopoulos, 1998). In all instances, results are expressed as mean ± S.E.M. Statistical analyses were performed by one-way ANOVA using Prism 4.03, and statistical significance was taken as \(p < 0.05.\)

**Results**

**Effect of Receptor Concentration on Saturation Binding of [3H]NMS.** Saturation binding experiments using three different receptor-membrane concentrations were performed to characterize the binding properties of the orthosteric probe [3H]NMS (Fig. 1). In general, the degree of ligand depletion was <10% for the 10 μg protein/tube concentration but approached 25% for the experiments using higher concentrations of receptor. The data sets obtained were analyzed using both a nondepletion (eq. 1) and a ligand depletion (eq. 2) saturation binding model.

As shown in Table 1, increasing the receptor-membrane concentration led to a decrease in the negative logarithm of the radioligand equilibrium dissociation constant (pK_A) when fitted with the nondepletion saturation model. This decrease in pK_A was significant for the highest receptor-membrane concentration (\(p < 0.05;\) one-way ANOVA). In contrast, the pK_A estimates obtained with the ligand depletion saturation model were more resistant to change in receptor-membrane concentration. The estimated maximal density of binding sites (B_max) also increased significantly with increasing receptor-membrane concentration for both models, as expected due to the increase in the amount of membranes added. For both versions of the binding model, the resulting best-fit curves were virtually superimposable (compare solid with dashed curves in Fig. 1) because both models contain enough parameters to adequately describe a sigmoid log concentration-occupancy relationship. Thus, goodness-of-fit cannot be used to discriminate between models in such a situation.

Although these findings illustrated the reliability of the ligand depletion saturation model, especially with respect to pK_A estimation at high receptor concentrations, the maximal levels of depletion observed did not exceed 25%. To more effectively probe the impact of depletion on competition and allosteric model binding parameters, the protein concentrations were increased to 15, 75, and 150 μg of protein for all subsequent experiments.

**Effect of Receptor Concentration on the Competition Binding of Orthosteric Ligands.** [3H]NMS competition binding experiments with the orthosteric agonist carbachol and antagonist atropine were performed using three different receptor-membrane concentrations. Inspection of the raw data revealed that radioligand depletion was less than 10% for 15 μg of protein, 25 to 30% for 75 μg of protein, and 35 to 40% for 150 μg of protein in the absence of the unlabeled orthosteric competitive ligands. The data sets obtained were fitted using the nondepletion (eq. 3) and ligand depletion (eq. 4) one-site competition binding equations (Fig. 2). The binding parameters obtained are given in Table 2.

When the data were fitted with a competition binding equation that does not assume depletion, increasing the receptor-membrane concentration decreased the estimated affinity for atropine, which was statistically significant at 150-μg protein concentration. In contrast, fitting the same data to the ligand depletion competition binding equation led to atropine affinity values that were essentially equivalent for each concentration of membrane (Table 2), demonstrating that this analysis was more appropriate for the assay conditions. With the agonist, carbachol, increasing the receptor concentration resulted in a decrease in estimated affinity values that was significantly different at the highest receptor-membrane concentration, irrespective of the model used to analyze the data. This latter finding suggests that there may be practical limitations to the depletion model(s).

**Effect of Receptor Concentration on the Binding of Allosteric Modulators.** Because the effects of ligand depletion on ATCM binding parameters have not been characterized, simulations were first performed to explore the possible effects that may be observed under such conditions. Figure 3 illustrates the effect of increasing receptor concentration for an allosteric enhancer and an inhibitor (data sets generated using eq. 7). The theoretical data sets obtained were then refitted using the nondepletion ATCM (eq. 5), and the allosteric ligand binding parameters (pK_A and log α) obtained were plotted against the theoretical receptor concentration used for the simulations (Fig. 4). As can be seen with the allosteric enhancer under the chosen simulation conditions, increasing the receptor concentration leads to an underestimation of cooperativity and overestimation of affinity due to

![Figure 1](image-url)
radioligand depletion (Fig. 4A). The opposite effect is seen in the presence of the post-competitive modulator (Fig. 4B). The [R]_T obtained on refitting the theoretical curves using the nondepletion ATCM was also plotted against the theoretical [R]_T used for the simulations (Fig. 5). The estimated [R]_T values using the nondepletion ATCM reach a plateau at receptor concentrations that were 10 times or higher than the added radioligand concentration, after which reliable estimates of [R]_T are not attainable.

It was reasoned that ligand depletion is more likely to occur with allosteric enhancers because the effect of these modulators is to increase the affinity of the orthosteric probe. Thus, additional simulations were performed to investigate the impact of varying the radioligand concentration, and the theoretical data sets were fitted using the nondepletion ATCM. Note that when compared with the pattern obtained in Fig. 4A, the estimates of pK_B and log α using the standard (nondepletion) ATCM with different radioligand concentrations demonstrated far less predictable behavior depending on the radioligand concentration (Fig. 6). This was manifested as highly spurious increases in the cooperativity factor to values such as log α = 20 in some cases, with corresponding pK_B estimates that were less than zero. In some cases, the nonlinear regression algorithm failed to converge altogether. These effects manifested at radioligand concentrations equal to or greater than the K_B value and when the total receptor concentration was greater than half the concentration of added radioligand. In contrast, the pattern of change in the pK_B and log α values obtained with allosteric inhibitors remained the same as shown previously in Fig. 4B irrespective of the concentration of radioligand (data not shown). These findings highlight the statistical correlation between pK_B and log α parameter estimates in the ATCM and the difficulty faced by the nonlinear regression algorithm in fitting such parameters under conditions where the (nondepletion) model simply cannot describe the data.

To test the reliability of the ligand depletion version of the ATCM, three theoretical data sets for an allosteric enhancer and an allosteric inhibitor each were generated using this model with varying [R]_T; to keep the emphasis on routine screening approaches, the number of data points for the curves and for all subsequent experiments were purposefully kept minimal, sampled only at logarithmic intervals. The data sets were then refitted with the ligand depletion equation (Fig. 7). Not surprisingly, the estimated allosteric ligand binding parameter estimates were the same as the theoretical values used for simulations, irrespective of the receptor concentration. In theory, this suggests that the ligand depletion ATCM is able to fit the data sets with very few data points and at a receptor concentration that is 10 times higher than the radioligand concentration used, where the ligand depletion reaches up to 90% in the absence of modulators. In practice, this is highly unlikely because of issues with biological variability and data quality. Nonetheless, it is clear that an ATCM that accounts for ligand depletion (at least up to 50%) can prove more reliable than one that does not.

Subsequently, radioligand binding experiments were performed to test and validate the ligand depletion ATCM. Alcuronium was used as the allosteric enhancer, and gallamine was used as the allosteric inhibitor. Ligand depletion in the absence of allosteric modulators ranged from less than 10% with the lowest membrane concentration to approximately 40% with the highest receptor-membrane concentrations. In

### Table 1

<table>
<thead>
<tr>
<th>Protein</th>
<th>Log B_max</th>
<th>pK_a</th>
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<tbody>
<tr>
<td>Nondepletion Saturation Model</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 μg</td>
<td>-10.46 ± 0.04</td>
<td>9.41 ± 0.13</td>
</tr>
<tr>
<td>50 μg</td>
<td>-9.69 ± 0.04*</td>
<td>9.11 ± 0.10</td>
</tr>
<tr>
<td>100 μg</td>
<td>-9.41 ± 0.05*</td>
<td>8.96 ± 0.07*</td>
</tr>
</tbody>
</table>

Logarithm of the maximal density of binding sites.

Values represent the mean ± S.E.M. of four experiments performed in duplicate.
the case of alcuronium, at the peak of the curve, ligand depletion ranged from approximately 15% for the lowest receptor-membrane concentration to approximately 50% for highest receptor-membrane concentration. The data sets were fitted with both the nondepletion ATCM and ligand depletion ATCM (Fig. 8). The estimated allosteric ligand binding parameters are given in Table 3.

With alcuronium, increasing the receptor-membrane concentration had a dramatic effect on the binding parameters obtained when the data were fitted using the nondepletion ATCM. In particular, a significant decrease in cooperativity was obtained upon increasing the receptor-membrane concentration. A decrease in affinity was also seen, but this was not significant. In contrast, when the data sets for alcuronium were fitted with the ligand depletion ATCM, the affinity and cooperativity values obtained for the different receptor-membrane concentrations were generally very similar, with the exception of the cooperativity estimate using the highest receptor-membrane concentration (Table 3). With gallamine, as expected, a significant increase in the cooper-
Activity estimate was obtained with 150 μg of protein when the data were fitted using the nondepletion ATCM. The allosteric ligand binding parameters obtained using the ligand depletion ATCM were more consistent across different receptor-membrane concentrations. Overall, the allosteric ligand binding parameters estimated using the ligand depletion ATCM were more consistent across experiments with different membrane concentrations.

Discussion

This study has found that significant depletion of the orthosteric antagonist probe, [3H]NMS, can lead to errors in the estimates of affinity and cooperativity of allosteric modulators of the M2 mAChR if the standard ATCM is applied to the data. This problem is worse with allosteric enhancers relative to inhibitors because the former agents can predispose the system toward orthosteric ligand depletion by enhancing the affinity of the probe. We have also developed and applied a version of the ATCM that specifically accounts for ligand depletion and have found that this model provides more reliable estimates of affinity for the modulators, alcuronium and gallamine, than the standard model under depletion conditions.

The development of high-throughput assays invariably necessitates addressing the issue of sensitivity. This could be related to sensitivity of the chosen analytical instrument or associated with the assay procedure itself. An obvious solution to the sensitivity issue is to increase the total amount of active biological product that generates the signal being measured. With enzymatic assays, this can be achieved by increasing the incubation time. Another way of amplifying the signal generated by the desired biological end product is to use a secondary enzyme-based reaction, such as in many enzyme-linked immunosorbent assays. However, with equilibrium radioligand binding assays, the desired signal is a

![Fig. 5.](image)

Fig. 5. Plots of the estimated log[R]T values obtained for an allosteric enhancer (o) and an allosteric inhibitor (○) on refitting the theoretical simulation curves (Fig. 3) using the nondepletion ATCM. Log[R]T on the x-axis represents the logarithm of the theoretical B_max values used for simulations.

![Fig. 6.](image)

Fig. 6. Estimates of pK_A (A) and log α (B) values obtained for an allosteric enhancer on refitting the theoretical simulation curves using the equilibrium ATCM. Log[R]T on the x-axis represents the logarithm of the theoretical B_max values used for simulations. Ligand depletion ATCM parameters used for the simulation were as follows: pK_A = 11.0, log α = 1.0, pK_B = 6.0, and NS = 0.0.

![Fig. 7.](image)

Fig. 7. Randomly generated data sets based on the ligand depletion ATCM and refitted using the same equation. A, allosteric enhancer. B, allosteric inhibitor. Parameters used for simulation were as follows: log α = 1.0 (enhancer) or −1.0 (inhibitor), pK_A = 11.0, log[A] = −12.0, pK_B = 6.0, and NS = 0.0.

![Fig. 8.](image)
function of the amount of receptors and the ligand concentration(s). Increases in amount of receptor without increases in volume will lead to an increase in receptor concentration, which can thus result in ligand depletion for high-affinity radioligands.

Under conditions of ligand depletion, the standard assumption underlying routine analyses of both orthosteric and allosteric binding events, i.e., that the free concentration of radioactive probe in the assay system should not be significantly different from the total probe added, no longer applies. For orthosteric ligand interactions, this has been demonstrated to lead to significant errors in the estimation of model parameters (Wells et al., 1980; Swillens, 1995). Although not the main focus of the current study, similar effects were noted in our study. The results with [3H]NMS and atropine clearly showed that increases in receptor concentration cause significant ligand depletion such that ligand affinity values were underestimated when using an equation that does not account for depletion. In contrast, the ligand depletion versions of saturation and one-site competition equations yielded orthosteric ligand binding parameters that were more consistent. It is interesting to note that the ligand depletion equation could still not compensate for the apparent reduction of agonist binding affinity at the highest receptor concentration. The reasons for this are unclear but could reflect a number of phenomena. For instance, mACHR agonists such as carbachol often reveal biphasic inhibition binding curves when tested against [3H]NMS, and ligand depletion has been shown to result in a progressive loss of the high-affinity parameter estimate (Wells et al., 1980). Because our study specifically wanted to mimic conditions representative of a screening assay rather than a detailed binding assay, few data points were used to generate the binding curve, and so resolution of two affinity states would not be meaningful. Thus, the apparent reduction in carbachol affinity in a one-site fit may be a mixture of differential effects on two affinity states in a two-site fit.

It is noteworthy that the current study has now demonstrated that probe depletion also leads to analytical problems with ATCM parameter estimation, especially with allosteric enhancers. This is understandable because positive cooperativity increases affinity of the orthosteric probe such that it may predispose it to ligand depletion even if the assay was initially designed to avoid that situation. More disturbingly, the application of the standard ATCM to allosteric enhancer data was clearly fraught with methodological pitfalls. For instance, the simulations suggested a general trend toward underestimation of cooperativity and overestimation of affinity. This would be expected for the following reasons. Ligand depletion would result in a system that behaved as if the apparent local concentration of orthosteric probe is very high; according to the ATCM, an increase in the concentration of orthosteric ligand would cause the resulting binding curve to approach the plateau value defined by $\alpha$; as such, the enhancement would be difficult to see above the already high degree of radioligand binding. However, if the concentration of radioligand (relative to its $K_B$) is not explicitly adjusted in the computerized analysis to account for this increase in local concentration, then the ATCM can only fit the same data by assuming that $\alpha$ is much lower than it actually is; in turn, because the parameters $K_B$ and $\alpha$ are correlated in a statistical (nonlinear model) sense, the affinity parameter will be overestimated to compensate for the cooperativity parameter being underestimated. Unfortunately, the simulations also indicated a more dire situation, i.e., there are conditions for allosteric enhancers where the standard ATCM would not converge at all to a satisfactory result. In contrast, ligand depletion is less problematic for allosteric inhibitors in a curve-fitting sense; the issues in this instance are essentially the same as those of orthosteric interactions under depletion conditions (i.e., underestimation of affinity) with the additional problem that an underestimation of $pK_B$ in the ATCM will lead to an overestimation of $\log \alpha$.

These observations were generally borne out when experiments were performed where ligand depletion was promoted by increasing the receptor concentration. As predicted, for the inhibitor gallamine, a decrease in apparent affinity and increase in apparent cooperativity was observed with increasing receptor concentrations when using the standard (nondepletion) ATCM. For alcuronium, a decrease in both affinity and cooperativity was noted, highlighting the general unreliability of the standard ATCM for enhancers under depletion conditions. However, when the data were analyzed using the ligand depletion ATCM, the parameters derived were more consistent across experiments with different receptor-membrane concentrations. Furthermore, the affinity and cooperativity estimates from the ligand depletion ATCM were in agreement with parameters derived using the nondepletion ATCM under nondepletion conditions (i.e., 15 μg of
The ligand depletion ATCM can be reliably used to derive allosteric ligand binding parameters. Although it may be argued that the actual over-/under-estimations in ATCM model parameters observed in our actual experiments were relatively modest, it should be noted that the simulations, which examined a wider range of system variables, identified the potential for larger discrepancies to be noted.

In practice, of course, the ideal situation is to avoid ligand depletion. In a screening sense, this may not be possible, especially if a very strong allosteric enhancer is identified. In that situation, the ligand depletion ATCM is preferable but still requires binding equilibrium at high modulator concentrations. This condition is not always attainable considering that some allosteric modulators can slow dissociation kinetics (Avlani et al., 2004). Care should be taken with such allosteric enhancers because the estimation of the cooperativity factor depends on the maximal plateau phase of the binding curve, which may not be clearly defined due to kinetic artifacts seen at very high modulator concentrations. In such cases, the binding parameters obtained will not be reliable, irrespective of the model used (depletion versus nondepletion).

There are additional considerations that have bearing on the application of this approach to the analysis of allosterism in radioligand binding assays. The first is that the model does not assume, and indeed cannot accommodate, any significant depletion in the concentration of unlabeled ligand, i.e., the allosteric modulator. The second is that an unknown amount of radioligand may be lost from the system as part of the filtration process, and this is not accounted for in the model. Although this may be a problem for low-affinity radioligands, we do not believe that this has a significant bearing in the current situation because ligand depletion is principally a problem with high (i.e., subnanomolar) affinity radioligands, which are not expected to undergo significant loss from the filters during rapid filtration (Yamamura et al., 1985). Third, if the allosteric modulator has additional agonist activity in its own right, then a complex binding interaction may result due to the propensity of the modulator to influence receptor-G protein coupling, which in turn will be influenced by receptor-G protein stoichiometry. This latter situation is similar to that encountered in studies of orthosteric agonist-antagonist interactions. Thus, biphasic modulator binding isotherms and/or sensitivity to guanine nucleotides are phenomena indicative of a more complex receptor-ligand interaction that cannot by accommodated by the ATCM used in the current study.

Taken together, the current studies highlight the impact of ligand depletion on the ATCM. It is shown that the effects of depletion are significantly greater for allosteric modulators compared with orthosteric ligands because they affect estimates of both affinity and cooperativity, even when depletion is relatively low (<20%). The ideal solution to the ligand depletion issue is to maintain assay conditions where there is no ligand depletion, but if that is not possible, then the ligand depletion equations can be used for estimating the ligand binding parameters when depletion is approximately <50%, provided that the system reaches equilibrium.

Table 3

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<th>Protein</th>
<th>Nondepletion ATCM</th>
<th>Ligand Depletion ATCM</th>
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<tr>
<td></td>
<td>( pK_a^a )</td>
<td>( \text{Log } \alpha )</td>
</tr>
<tr>
<td>Alcuronium</td>
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<tr>
<td>15 ( \mu )G</td>
<td>6.12 ± 0.21</td>
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* Significantly different \( (p < 0.05) \) compared with the parameters obtained for 15-\( \mu \)G protein concentration using the same equation.
† Significantly different \( (p < 0.05) \) compared with the values obtained using the ligand depletion ATCM with the same protein concentration.

\( \log \) Logarithm of the equilibrium dissociation constant for the allosteric modulator at the free receptor.

\( \alpha \) Logarithm of the cooperativity factor.

References


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