Activation of G protein-coupled receptors entails cysteine modulation of agonist binding

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Abstract

The increase in the affinity of agonists with an increase in pH and experiments using thiol-specific reagents indicate that G protein-coupled receptors contain an ionizable cysteine residue at the ligand binding site. Since treatment of receptors with reducing agents produces functional activation and potentiates agonist stimulation, it is likely that this free sulphydryl modulates receptor activation. We have derived a two-state acid–base model for cysteine modulation of ligand binding which leads to a description of ligand efficacy. We have shown that pH-dependent binding of agonists is closely correlated with measurements of ligand efficacy at the 5-HT2A receptor. In general, efficacy is determined by the preference of a ligand for the base form of the receptor. Efficacy may also be described in thermodynamic terms as the coupling free energy involving a ligand and the acid and base states of the receptor. Molecular modeling of the third transmembrane domain of the 5-HT2A receptor, which contains a conserved cysteine, shows that efficacy is determined by the difference between the electrostatic interaction energies of a ligand with the acid and base forms of the receptor model. The difference in interaction energy between the two forms of cysteine makes the largest contribution to this electrostatic interaction energy difference. Therefore, the cysteine makes the largest contribution to ligand efficacy. Using this approach, we can distinguish between the efficacies of agonists with varying molecular structures and account for the difference between the properties of agonists and antagonists. © 1998 Elsevier Science B.V.

Keywords: Free sulphydryl; Ligand efficacy; pH-dependent binding; Receptor activation; Two-state acid–base model © 1998 Elsevier Science B.V.

Abbreviations: ANOVA = analysis of variance; BUFO = 5-hydroxy-N,N-dimethyltryptamine (bufotenin); DTNB = 5,5′-dithiobis-2-nitrobenzoic acid; DTT = dithiothreitol; G protein = guanine nucleotide binding regulatory protein; 4HDM = 4-hydroxy-N,N-dimethyltryptamine; 4-HT = 4-hydroxytryptamine; 5-HT = 5-hydroxytryptamine (serotonin); 5-MOT = 5-methoxytryptamine; G<sub>s</sub> = stimulatory GTP-binding regulatory protein of adenylcyclase; RIE = relative intrinsic efficacy; NMDA = N-methyl-d-aspartate; MDDM = 4,5-methylenedioxo-N,N-dimethyltryptamine; MDOX = 4,5-methylenedioxy-tryptamine; PCMB = p-chloromercuribenzoic acid; TRDM = N,N-dimethyltryptamine; TRYP = tryptamine

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1. Introduction

The interaction of an agonist with a cell surface receptor can lead to transmission of information across the cell membrane. One type of system which conveys this information contains, in addition to the ligand and the receptor protein, a G protein and an effector protein [1]. Attempts to understand the connection between binding and activation have led to the idea of efficacy [2], which is a measure of the response associated with ligand binding. In essence, efficacy measures a molecular property of the ligand which perturbs the receptor and alters its interaction with another protein [3].

Activation has been described in terms of a ternary complex model [4]. This model does not account for constitutive activity and offers no explicit molecular description of how the interaction between agonist and receptor produces coupling with the G protein. Agonists display increased affinities for constitutively active mutant receptors compared with wild-type receptors [5,6]. This affinity increase is proportional to agonist efficacy even under conditions where coupling of the receptor with G protein is unlikely. The mutations associated with constitutive activity give rise to a high-affinity G protein-independent state of the receptors. As a result, the ternary complex model was extended to include an explicit isomerization of the receptor R to an active state R* [5,6]. We assume that this active state is similar to the state which is induced by an agonist during activation of the wild-type receptor.

There is no detailed molecular description of ligand efficacy. In fact, there is no clear distinction between the molecular properties of an agonist and an antagonist. However, there are some important clues which can be derived from an analysis of pH-dependent binding and response. Additional evidence comes from observations that DTT and other reducing agents produce functional activation in the presence or absence of agonists.

It was observed for a number of G protein-coupled receptors that the binding of agonists shows a distinct pH-dependence [7–14]. In each case, the affinity of an agonist increases with increasing pH, unless the increase in pH deprotonates the ligand. Except for substituted benzamides on the D2 receptor [12,14], antagonist binding shows little or no pH-dependence in the pH range 6–8 [7,12,13]. For the 5-HT2A receptor, experimental data are available for both the pH-dependence of agonist binding and ligand efficacies. The pH-dependence of binding was measured for a series of tryptamine analogs at 5-HT2A receptors labeled by [3H]-ketanserin [7]. In addition, the relative intrinsic efficacies (RIE) of these ligands were determined using response data obtained from the rabbit aorta [15,16].

The change in the affinity of agonists at the 5-HT2A receptor is not due to a change in their ionization states. Since the pKₐ values of these agonists are greater than 9 [17], it is likely that within the experimental pH range, 7.0–8.2, they are predominantly in the cation form. An alternative explanation for the pH-dependence of agonist binding is that the binding site of the receptor contains one or more ionizable residues with pKₐ values within or near the experimental pH range. Two amino acids with pKₐ values near this range are cysteine and histidine. The acid form of cysteine is electrically neutral and the base form is negatively charged. On the other hand, the acid form of histidine is positive and the base form is neutral. Since the affinity of an agonist increases with increasing pH, it is likely that the positively-charged agonist interacts with a receptor amino acid which changes from neutral to negative as the pH increases. Thus, cysteine may be considered the more likely amino acid which modulates the pH-dependent affinity of an agonist. Since a free cysteine can exist in both acid and base forms, there is a straightforward connection to the experimentally observed pH-dependent binding.

The addition of DTT or other thiol compounds causes functional activation of receptors in the presence or absence of agonist ligands [18,19]. When purified β-adrenergic receptors and purified Gs were inserted in phospholipid vesicles, treatment of the receptor with DTT prior to reconstitution gave rise to G protein activation even in the absence of agonist [18]. There was no effect of DTT on Gs alone, demonstrating that the receptor itself was the target of the reducing agent. Thus, free sulfhydryl groups are likely to be critical components in the process of receptor activation. In addition, DTT potentiated the activation produced by an agonist. This implies that agonists interact with free sulfhydryls in the course of activation. DTT also produced
functional activation and potentiated agonist activation at muscarinic receptors [19]. In related experiments, DTT potentiates the response of the NMDA receptor, whereas DTNB attenuated the response [20]. Although the NMDA receptor is not considered to be a G protein-coupled receptor, this and the previous evidence suggest that a free cysteine residue is essential for receptor activation.

Alkylating reagents such as PCMB or DTNB, which react with free thiol groups, were shown to affect both ligand binding [21–29] and receptor activation [24]. In several of these experiments, thiols could be protected from alkylation when the receptor was previously occupied by either antagonists or agonists [22–26,28]. The effects of these alkylating reagents could be reversed by the reducing agent, DTT [21,24,27,28]. Taken together, these results are consistent with the presence of a free sulfhydryl group in the vicinity of the ligand binding site.

In the rhodopsin receptor, Cys$^{110}$ and Cys$^{187}$, which correspond to conserved cysteines in the G protein-coupled receptors, were considered to exist as components of a disulfide linkage. However, recent evidence from Fourier transform infrared difference spectroscopy indicates the presence of a free sulfhydryl group during the course of photoactivation [30].

In order to determine the likely location of a free cysteine in G protein-coupled receptors, we have referred to the amino acid sequences of these receptors [31]. Each sequence contains two conserved cysteine residues in extracellular regions. Mutations of $\beta_2$-adrenergic receptors confirm the importance of these conserved cysteines. Mutation of either Cys$^{106}$ or Cys$^{184}$ to either Ala [32] or Val [33] reduced the adenylate cyclase activity. In each case, the Cys$^{106}$ mutation had a greater effect on activation. When Cys$^{184}$ was mutated to Val, the activity was reduced, but approximately 60% of the wild-type activity was retained [34]. From these observations, it appears that Cys$^{106}$, which is located at the extracellular end of the third transmembrane helix, is the major contributor to receptor activation.

From the analysis of the pH-dependent binding, the functional activation by DTT and the alkylation experiments, we hypothesize that there exists at least one ionizable cysteine which modulates ligand binding and receptor activation. We have tested this hypothesis by analyzing the acid–base equilibrium associated with a cysteine residue in a detailed molecular model for the binding site of the 5-HT$^{2A}$ receptor.

2. Methods

2.1. Two-state model for the acid and base forms of the receptor

In order to examine the relationship between the pH-dependent binding and efficacy, we derived the following two-state model for the acid and base forms of the receptor.

1. The receptor is assumed to exist in acid, $R_A$ and base, $R_B$, forms corresponding to the acid and base forms of a cysteine residue;

2. Since the binding of the radiolabeled antagonist ligand, $L$, shows only a slight pH-dependence, it is assumed that the antagonist has the same affinity for both forms of the receptor;

$$LR_A \rightleftharpoons L + R_A \quad K_{LA} = \frac{[L][R_A]}{[LR_A]}$$

$$LR_B \rightleftharpoons L + R_B \quad K_{LB} = \frac{[L][R_B]}{[LR_B]}$$

$$K_{LA} = K_{LB} = K_L;$$

3. The competing agonist, $D$, has different affinities for the two forms of the receptor;

$$DR_A \rightleftharpoons D + R_A \quad K_{DA} = \frac{[D][R_A]}{[DR_A]}$$

$$DR_B \rightleftharpoons D + R_B \quad K_{DB} = \frac{[D][R_B]}{[DR_B]}$$

4. We define the total number of receptors in each form where the total receptors in acid and base form are linked by an equilibrium constant, $K_R$;

$$R_{TOTAL_A} = R_A + LR_A + DR_A, \quad R_{TOTAL_B} = R_B + LR_B + DR_B.$$
The total receptors in acid and base form are linked by an equilibrium constant, $K_R$:

\[
(R_{\text{TOTAL}})_A \rightleftharpoons (R_{\text{TOTAL}})_B + H^+
\]

(1)

\[
K_R = \frac{[(R_{\text{TOTAL}})_B][H^+]}{[(R_{\text{TOTAL}})_A]}
\]

5. The assumption is made that once ligands L and D are in equilibrium with the receptor, there is no net interconversion between the acid and base forms of the receptor—the binding of a cationic ligand stabilizes the ionized form of the cysteine residue;

6. It is also assumed that the value of $K_R$ is independent of the pH.

2.2. Fitting method I: determination of $K_{DA}$, $K_{DB}$ and $K_R$ from values of $IC_{50}$ at several pH values

The amount of L which is bound to the two forms of the receptor in the presence of the competing ligand, D, is given by:

\[
\frac{(R_{\text{TOTAL}})_A[L]}{[L] + K_L(1 + [D]/K_{DA})} + \frac{(R_{\text{TOTAL}})_B[L]}{[L] + K_L(1 + [D]/K_{DB})}
\]

When $[D] = IC_{50} = D_{50}$:

[amount of L bound (with D)]

\[
= \frac{1}{2} \text{[amount of L bound (with no D)].}
\]

Therefore:

\[
\frac{2(R_{\text{TOTAL}})_A}{[L] + K_L(1 + D_{50}/K_{DA})} + \frac{2(R_{\text{TOTAL}})_B}{[L] + K_L(1 + D_{50}/K_{DB})}
\]

\[
= \frac{(R_{\text{TOTAL}})_A + (R_{\text{TOTAL}})_B}{[L] + K_L}
\]

Since (see Eq. (1)) $(R_{\text{TOTAL}})_B = (R_{\text{TOTAL}})_A (K_R/[H^+])$, the previous equation, after some rearrangement, becomes:

\[
\frac{2[H^+]}{[L] + K_L(1 + D_{50}/K_{DA})} + \frac{2K_R}{[L] + K_L(1 + D_{50}/K_{DB})}
\]

\[
= \frac{[H^+] + K_R}{[L] + K_L}
\]

If this equation is solved for $K_R$, the result is:

\[
K_R = -\frac{[H^+](\lambda + D_{50}/K_{DB})(\lambda - D_{50}/K_{DA})}{(\lambda + D_{50}/K_{DA})(\lambda - D_{50}/K_{DB})},
\]

(2)

where $\lambda = [L]/K_L + 1$.

For each agonist, D, values of $K_{DA}$ and $K_{DB}$ were changed in steps until the standard deviation of the values of $pK_R$ (see Eq. (2)), calculated from the IC$_{50}$ values at several pH values, was minimized.

2.3. Fitting method II: determination of $pK_R$, $K_{DA}$ and $K_{DB}$ from points read from competition binding curves (fraction radioligand antagonist bound versus concentration of competing agonist, D) at several pH values

Parameters $pK_R$, $K_{DA}$ and $K_{DB}$ were fitted to competition binding curves at several pH values. A given value of $pK_R$ was combined with each experimental pH value to calculate the fractions of acid and base states in the receptor (see Eq. (1)). Calculated values of fraction L that was bound were compared with experimental values. Values of the three parameters were changed in steps until the quantity [calculated fraction bound – experimental fraction bound]$^2$ was minimized.

2.4. Thermodynamic description of the two-state acid–base model

The two-state acid–base model may be expressed in thermodynamic form. Each of the equilibrium constants involving the binding of the agonist, D, may be linked to the corresponding change in the Gibbs free energy. For the acid and base forms, these are:

\[\Delta G_A = -RT \ln K_{DA}\]

\[\Delta G_B = -RT \ln K_{DB}.\]

Assuming that an ionizable residue in the receptor accounts for the pH-dependence of binding, we define a coupling free energy [36] which is given by:

\[\Delta \Delta G^\circ = (\Delta G_A^\circ - \Delta G_B^\circ)\]

in terms of the dissociation constants of the agonist, the coupling free energy is expressed by

\[\Delta \Delta G^\circ = -RT \ln(K_{DA}/K_{DB}).\]
2.5. Molecular modeling of receptor–ligand interactions at the 5-HT$_2A$ receptor

The program QUANTA was used to build the receptor model; it consisted of 31 residues from the third transmembrane domain (TM3) with the conserved cysteine residue in either the acid or the base form. The 31 residues correspond to the sequence Ser$^{145}$–Val$^{175}$ in the 5-HT$_2A$ receptor [35].

Residue no. 4 corresponds to Cys$^{148}$. All hydrogen atoms were included explicitly. The secondary structures were as follows: residues 1–2 (antiparallel beta); residues 3–28 (alpha helix); residues 29–31 (antiparallel beta). Standard CHARMM charges were used for all receptor atoms except for the base form of cysteine in which case the charges were derived from a calculation of the molecule ethyl sulfide with a 6–31G basis set. The calculated charges were then scaled and adapted to CHARMM format.

Ligand models in the form of cations were built with the program QUANTA. Charges were determined from calculations with an STO-3G basis set. The receptor–ligand complexes were prepared by docking the ligand, using the facilities of QUANTA, into the acid form of the receptor with cysteine in the SH form. After each tryptamine analog ligand was docked, the side chain nitrogen atom was near the ASP residue of the receptor, the aromatic indole ring was approximately parallel to the plane of the TRP residue and the N–H group of the indole ring was near the CYS residue (see Fig. 1). The docked position of the ligand obtained with the acid form was also used as the starting position for the base form. The energy of each form of the receptor–ligand complex was then minimized using the adopted basis Newton–Raphson (ABNR) algorithm of CHARMM (version 21). A dielectric constant of 10.0 and a non-bonded cut-off distance of 15.0 Å were used for all runs. A run was considered acceptable when the root-mean-square difference of the ligand atom positions obtained from the acid and base minimizations was less than 0.5 Å. At least four runs were performed for each agonist. Five runs were performed for the antagonist, spiperone. Three runs were performed for the antagonist ketanserin.

For a minimized ligand–receptor complex, the interaction energy between the ligand and the receptor was calculated using the expression:

$$\Delta E = E_{RL} - E_R - E_L,$$

where $E_{RL}$ is the energy of the receptor–ligand complex, $E_R$ is the energy of the receptor and $E_L$ is the energy of the ligand.

The total interaction energy, $\Delta E$, was partitioned into Lennard–Jones and electrostatic terms according to the expression:

$$\Delta E = \Delta E^{\text{TOT}} = \Delta E^{\text{LJ}} + \Delta E^{\text{EL}}.$$

Interaction energies were also calculated between the ligand and each of five nearest neighbor residues. The difference between the interaction energies in base and acid form, designated as $\Delta \Delta E$, was calculated using the expression:

$$\Delta \Delta E = \Delta E^{\text{BASE}} - \Delta E^{\text{ACID}}.$$
versus pH yields a linear relationship between binding and pH [Fig. 2(A)]. The relative intrinsic efficacies (RIE) of these ligands were determined using response data from the rabbit aorta [15,16]. The slopes of the lines from Fig. 2(A) correlate with the values of RIE [Fig. 2(B)]. Thus, there appears to be a coherence between the pH-sensitive binding of a ligand and its efficacy.

3.2. Analysis of experimental data using the two-state acid–base model

The pH-dependent binding data for four drugs (5-hydroxytryptamine, 5-methoxytryptamine, tryptamine and bufotenin) at the 5-HT$_{2A}$ receptor was analyzed according to the two-state model using fitting method I (see Section 2).

The results are presented in Table 1, along with the values of RIE obtained from measurements on the rabbit aorta [15,16]. For 5-hydroxytryptamine, competition curves calculated from the fitted parameters $pK_R$, $K_{DA}$ and $K_{DB}$ are in close agreement with the experimental competition curves. The root-mean-square differences between calculated and experimental values of fraction radioligand bound are 0.023, 0.017, 0.041 and 0.052, at the pH values 7.0, 7.4, 7.8 and 8.2, respectively (data not shown). For each pH value, the two-state acid–base fit is significantly better than a one-site fit ($F$-test; $p < 0.05$).

For each of the four agonists, values of the Hill slopes at four pH values were determined from the competition curves generated by using the fitted values of $pK_R$, $K_{DA}$ and $K_{DB}$. In each case, the lowest Hill slope occurs at the pH value of 7.4 which is closest to the fitted value of $pK_R$. If the pH were equal to $pK_R$, the populations of acid and base receptor states would be equal. Average values of the Hill slopes (Table 1) calculated at four pH values are significantly correlated with the values of $\Delta \Delta G^0$ which were derived (see Section 2) from the fitted values of $K_{DA}$ and $K_{DB}$ ($r = 0.999$, $p < 0.002$). The average values of the Hill slopes are also significantly correlated with the experimentally determined values of RIE ($r = -0.96$, $p < 0.005$). Interestingly, the fitted values of $pK_R$ for the four agonists are not significantly different (ANOVA; $0.10 < p < 0.25$). Assuming that the value of $pK_R$ corresponds to the ionization constant of a cysteine residue located near the binding.

3. Results and discussion

3.1. The pH-dependence of ligand binding and its connection with efficacy

The pH-dependence of ligands binding to 5-HT$_{2A}$ receptors labeled by [3H]-ketanserin was measured at the experimental pH values of 7.0, 7.4, 7.8 and 8.2 [7]. The affinities of antagonists varied only slightly with change in pH. In contrast, the affinities of agonists showed a distinct increase with increasing pH. For each of four tryptamine analogs, a plot of log IC$_{50}$

![Graph A](image1)

Graph A: pH-Dependence of binding for four tryptamine analogs at 5-HT$_{2A}$ receptors labelled by [3H]-ketanserin. (A) Plots of log IC$_{50}$ versus pH. The regression equations are: 5-HT log IC$_{50} = 1.01-1.03$ pH ($r = -0.993$; $p < 0.01$); 5-MOT log IC$_{50} = 0.710-0.991$ pH ($r = -0.997$; $p < 0.005$); TRYP log IC$_{50} = 0.174-0.813$ pH ($r = -0.98$; $p < 0.02$); BUFO log IC$_{50} = -1.46-0.712$ pH ($r = -0.96$; $p < 0.05$). (B) Plot of the slopes of fitted lines from (A) versus relative intrinsic efficacies (RIE) from refs. 15 and 16. Regression equation: slope = $-0.627-0.407$ RIE ($r = 0.997$; $p < 0.005$).
Table 1
Two-state acid–base model for pH-dependent agonist binding at 5-HT2A receptors

<table>
<thead>
<tr>
<th>Agonist</th>
<th>$K_{DA}$ (nM)$^a$</th>
<th>$K_{DB}$ (nM)$^a$</th>
<th>$K_{DA}/K_{DB}$ (nM)</th>
<th>$\Delta\Delta G^\circ$ (kcal mol$^{-1}$)$^c$</th>
<th>$pK_R$ (kcal mol$^{-1}$)$^d$</th>
<th>RIE$^c$</th>
<th>Hill$^f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT</td>
<td>646</td>
<td>19.5</td>
<td>33.1</td>
<td>$-2.16$</td>
<td>$7.49783 \pm 0.00020$</td>
<td>1.00</td>
<td>$0.790 \pm 0.023$</td>
</tr>
<tr>
<td>5-MOT</td>
<td>558</td>
<td>17.5</td>
<td>31.9</td>
<td>$-2.13$</td>
<td>$7.529 \pm 0.052$</td>
<td>0.86</td>
<td>0.794 $\pm 0.022$</td>
</tr>
<tr>
<td>TRYP</td>
<td>2464</td>
<td>148.5</td>
<td>16.6</td>
<td>$-1.73$</td>
<td>$7.545 \pm 0.064$</td>
<td>0.48</td>
<td>0.859 $\pm 0.020$</td>
</tr>
<tr>
<td>BUFO</td>
<td>392</td>
<td>26.5</td>
<td>14.8</td>
<td>$-1.66$</td>
<td>$7.39 \pm 0.16$</td>
<td>0.20</td>
<td>0.867 $\pm 0.024$</td>
</tr>
</tbody>
</table>

$^a$Step-size used in iteration = 1 nM.
$^b$Step-size used in iteration = 0.5 nM.
$^c$Values calculated at a temperature of 310 K. Regression equation for $\Delta\Delta G$ versus relative intrinsic efficacy (RIE): $\Delta\Delta G = -1.48 - 0.699$ RIE ($r = -0.97; p < 0.05$).
$^d$Values are the arithmetic mean $\pm$ standard deviation. Average value of $pK_R$ for four agonists is 7.491 $\pm 0.070$.
$^e$Values taken from Refs [15,16].
$^f$Average value of Hill slopes ($\pm$ standard deviation) calculated at pH values 7.0, 7.4, 7.8 and 8.2.

site, one may expect that the ionization is affected by the agonist and the neighboring residues in the receptor. Since, in this case, the four agonists are structurally similar, the observed agreement among the values of $pK_R$ is quite reasonable.

There is a highly significant correlation between the fitted values of $K_{DE}$ and the experimental values of $EC_{50}$ [15,16] ($r = 0.998; p < 0.005$). Thus, the affinity of an agonist for the base form of cysteine is closely linked to activation. The fitted values for the ratio $K_{DA}/K_{DB}$ are significantly correlated with the experimentally determined efficacies ($r = 0.96; p < 0.05$). Comparison of the pH-dependent binding data [7] with activation data [15] shows that for four drugs acting at the 5-HT$_{2A}$ receptor there is a close correlation (see Table 1) between the calculated $\Delta\Delta G^\circ$ and the experimentally measured relative intrinsic efficacy ($r = -0.97; p < 0.05$). This close correspondence justifies the assumptions of the two-state acid–base model used in this analysis. An agonist with a high efficacy has a large magnitude for $\Delta\Delta G^\circ$ and demonstrates a strong preference for the base form

Table 2
Two-State acid–base model for pH-dependent agonist binding at other receptors

<table>
<thead>
<tr>
<th>(Receptor)/ligand</th>
<th>pH$^a$</th>
<th>$K_{DA}$ (nM)</th>
<th>$K_{DB}$ (nM)</th>
<th>$K_{DA}/K_{DB}$ (kcal mol$^{-1}$)</th>
<th>$\Delta\Delta G^\circ$ (kcal mol$^{-1}$)$^b$</th>
<th>$pK_R$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>(5-HT$_{2A}$ receptor)/</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HT$^c$</td>
<td>6.5–9.4</td>
<td>24.00</td>
<td>6.25</td>
<td>3.84</td>
<td>$-0.83$</td>
<td>7.60</td>
<td>[8]</td>
</tr>
<tr>
<td>8-OH-DPAT$^c$</td>
<td>6.5–9.4</td>
<td>33.50</td>
<td>9.25</td>
<td>3.62</td>
<td>$-0.79$</td>
<td>7.80</td>
<td>[8]</td>
</tr>
<tr>
<td>(\alpha$_2$ receptor)/</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epinephrine</td>
<td>6.5, 7.5</td>
<td>767</td>
<td>146.5</td>
<td>5.24</td>
<td>$-1.02$</td>
<td>6.90</td>
<td>[9]</td>
</tr>
<tr>
<td>(\beta$_2$ receptor)/</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>6.69, 7.65</td>
<td>5483</td>
<td>954</td>
<td>5.75</td>
<td>$-1.08$</td>
<td>6.5</td>
<td>[10]</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>6.69, 7.65</td>
<td>633</td>
<td>156.5</td>
<td>4.04</td>
<td>$-0.86$</td>
<td>6.7</td>
<td>[10]</td>
</tr>
<tr>
<td>(D$_1$ receptor)/</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCH23390</td>
<td>5.0–10</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>6.9$^d$</td>
<td>[11]</td>
</tr>
<tr>
<td>(D$_2$ receptor)/</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epidepride$^e$</td>
<td>6.8, 8.0</td>
<td>15.4</td>
<td>0.10</td>
<td>15.4</td>
<td>$-3.10$</td>
<td>7.46</td>
<td>[12]</td>
</tr>
<tr>
<td>Quinpirole</td>
<td>6.8, 8.0</td>
<td>7047</td>
<td>410.5</td>
<td>17.2</td>
<td>$-1.75$</td>
<td>7.34</td>
<td>[12]</td>
</tr>
<tr>
<td>Sulpiride$^e$</td>
<td>5.5–8.5</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>7.3$^d$</td>
<td>[14]</td>
</tr>
</tbody>
</table>

$^a$Experimental pH values or pH range.
$^b$Values calculated at a temperature of 310 K.
$^c$The $pK_R$ value for the ligand was included in the fit. For 5-HT, the fitted value for $pK_R$ is 9.00. For 8-OH-DPAT, the fitted value for $pK_R$ is 9.95.
$^d$Experimentally determined $pK_R$ value.
$^e$Ligand displays pH sensitivity because it contains a benzamide group which interacts with a modulating cysteine residue.
Table 3
Effect of guanyl nucleotides on binding of agonists at the 5-HT$_{2A}$ receptor; application of the two-state model$^{*}$

<table>
<thead>
<tr>
<th>Agonist</th>
<th>$K_{DA}$ (nM)$^b$</th>
<th>$K_{DB}$ (nM)$^c$</th>
<th>$\Delta \Delta G^\circ$ (kcal mol$^{-1}$)$^d$</th>
<th>$K_{DA}$ (nM)$^b$</th>
<th>$K_{DB}$ (nM)$^c$</th>
<th>$\Delta \Delta G^\circ$ (kcal mol$^{-1}$)$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT</td>
<td>5493</td>
<td>61.5</td>
<td>−2.77</td>
<td>10187</td>
<td>375.5</td>
<td>−2.03</td>
</tr>
<tr>
<td>5-MOT</td>
<td>4905</td>
<td>79.5</td>
<td>−2.54</td>
<td>7943</td>
<td>628.0</td>
<td>−1.56</td>
</tr>
<tr>
<td>TRYP</td>
<td>13205</td>
<td>396.5</td>
<td>−2.16</td>
<td>20526</td>
<td>2038.0</td>
<td>−1.42</td>
</tr>
<tr>
<td>BUFO</td>
<td>2490</td>
<td>102.0</td>
<td>−1.97</td>
<td>4285</td>
<td>505.0</td>
<td>−1.32</td>
</tr>
</tbody>
</table>

$^*$The experimental pH value was 7.4. A p$K_R$ value of 7.491 (derived from analysis of pH-dependent binding) was used in all calculations.

$^b$Step-size used in iteration = 1 nM.

$^c$Step-size used in iteration = 0.5 nM.

$^d$Values calculated at a temperature of 310 K.

of the cysteine over the acid form. In contrast, antagonist binding shows approximately equal affinities for the acid and base forms, implying that the ratio $K_{DA}/K_{DB}$ would be approximately equal to one and the corresponding value of $\Delta \Delta G^\circ$ would be close to zero.

The pH-dependent binding data for other receptors were also analyzed according to the two-state acid–base model using fitting method II (see Section 2). These results are presented in Table 2. The values of p$K_R$ determined for other receptors are somewhat different from those determined for the 5-HT$_{2A}$ receptor (compare Table 1) which is reasonable if one considers that the polarization of nearby residues tends to stabilize the charged form of cysteine. Thus, a receptor which contains highly polarizable neighboring residues would tend to have a reduced value for p$K_R$.

3.3. The pH-dependence of response

To test whether the pH-dependence of binding could also be observed in a system which measures response, preliminary experiments$^1$ were conducted with a rabbit aorta preparation which contains 5-HT$_{2A}$ receptors [15,16]. In the absence or presence of the agonist 5-HT, raising the pH by addition of NaOH produced an increase in the tension of the tissue, whereas lowering the pH by addition of HCl gave a decrease in the tension. Therefore, the contraction was pH-dependent even in the absence of agonist. These observations show that the activation process is intimately connected with the conversion of the receptor from acid to base form.

Other effects of moderate pH changes on smooth muscle tissues have also been reported [37]. Increases in pH produced contractions, whereas decreases in pH caused relaxations. Responses of whole cells to NMDA have also been reported to be pH-dependent [38]. An increase in pH caused potentiation of the response, whereas a decrease in pH caused a decrease in the response. The data for response to NMDA was analyzed according to a two-state model similar to the one we have presented, but no reference was made to a cysteine residue.

3.4. Competition curves and the two-state acid–base model: effects of guanine nucleotides

Competition curves at 5-HT$_{2A}$ receptors were also presented in another paper [39]. This study included the same four tryptamine analogs which had been used for pH-dependent binding [7]. We analyzed points from the experimental competition curves according to the two-state acid–base model. The average value of p$K_R$ obtained from the pH-dependent analysis was combined with the experimental pH value to calculate the fractions of acid and base states in the receptor. The values of $K_{DA}$ and $K_{DB}$ were varied to fit the experimental competition curves. Values of $\Delta \Delta G^\circ$ for four agonists were determined from the dissociation constants. The results, shown in Table 3, are similar to those determined independently from pH-dependent binding (compare Table 1). The values of $\Delta \Delta G^\circ$ are also closely correlated with the experimental values of RIE ($r = −0.99; p < 0.02$). Analysis of the competition curves in the presence of GTP gave

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$^1$ S. Maayani, private communication.
values of $\Delta \Delta G^o$ with magnitudes smaller than those obtained in the absence of GTP. The values of $K_{DA}$ and $K_{DB}$ were both increased in the presence of GTP. The value of $K_{DB}$ was increased by a larger factor, which indicates that the perturbation of $K_{DB}$ made the dominant contribution to the change in $\Delta \Delta G^o$ after treatment with GTP. Since GTP is necessary for the activation process, the observation of its pronounced effect on $K_{DB}$ shows the connection between the base form of the receptor and activation.

For each ligand, whether in the absence or presence of GTP, the two-state acid–base model fits the data better than a one-site model ($F$-test; $p < 0.05$). This implies that the two states are intrinsically associated with the ligand–receptor interaction and that these states persist even when the guanyl nucleotide has partially decoupled the receptor from the G protein.

The coupling free energy involving the ligand and the two forms of the receptor may be transferred to the G protein during the course of activation. Addition of guanine nucleotides, which partially decouples the receptor from the G protein, reduces the magnitude of $\Delta \Delta G^o$ and apparently decreases the transfer of this free energy. A negative value for $\Delta \Delta G^o$ implies that the two-state system in the agonist–receptor complex triggers a spontaneous process which can be used to drive an endergonic change associated with the G protein (e.g. the release of GDP).

The transfer of coupling free energy from the receptor to the G protein may involve electrostatic interactions between a charged cysteine and other charged residues in the receptor.

### 3.5. Molecular modeling of receptor–ligand interactions at the 5-HT$_{2A}$ receptor

All G protein-coupled receptors contain a conserved cysteine residue at the extracellular end of the third transmembrane helix. In the $\beta_2$-adrenergic receptor, Cys$^{106}$ was shown to have an important connection to activation [32–34]. In all of the neurotransmitter receptors, this cysteine is near a conserved aspartate residue in the third helix. Mutagenesis [40–44] and labeling [45] experiments have indicated that this aspartate residue forms a salt bridge with the amino side chain of the cation ligand. Mutation of Asp$^{113}$ in the $\beta_2$-adrenergic receptor to Asn or Glu drastically reduces ligand binding, but does not eliminate activation [46]. This suggests that a residue other than Asp is intrinsically involved in activation. Interestingly, the rank order for the EC$_{50}$ values for three agonists is the same in both mutant and wild-type receptors. In almost all of the neurotransmitter receptors, a functionally conserved aromatic residue is located between the cysteine and the aspartate [32]. It is likely that the aromatic ring which is found in many ligands interacts with this aromatic residue by means of dispersion interactions which strengthen ligand binding. Previous work on the 5-HT$_{2A}$ receptor established the importance of a tryptophan residue in making ring–ring interactions with the agonist 5-HT [47].

Since, from our analysis, the acid and base forms of cysteine are associated with both binding and activation, we sought a detailed molecular description of the interactions between the two states of cysteine and a ligand. Sequences for the helix 3 region of the 5-HT$_{2A}$
and other neurotransmitter receptors are shown in Fig. 3. Since the third helix in the 5-HT_{2A} receptor is the only region which contains the three important residues, Cys, Trp and Asp, in close proximity, we have constructed a three-dimensional model of the third transmembrane domain to analyze the detailed molecular interactions of ligands with the receptor. In order to gage the effect of the charge of the cysteine on the ligand, the model was constructed with cysteine in both acid and base forms.

The three-dimensional structure of the binding site (see Fig. 1) shows that the cysteine and aspartate lie on the same face of the helix about 10 Å apart. The functionally conserved aromatic residue also lies on the same face of the helix about midway between the cysteine and the aspartate. A ligand was docked in the model of the 5-HT_{2A} receptor as described in Methods. Each ligand interacted favorably within the region spanning the Cys and the Asp.

We have calculated the interaction energies for each of nine tryptamine analogs (see Fig. 4) with both acid and base forms of the 5-HT_{2A} receptor model. The set of analogs includes both full and partial agonists whose relative intrinsic efficacies were measured experimentally [15,16]. We have previously demonstrated from our analysis of pH-dependent binding that \( \Delta G^\circ \) correlates with efficacy. Assuming that these ligands, which are of similar size, make similar entropic contributions to the free energy of binding, we have used the differences between the calculated interaction energies in the acid and base forms to represent the differences between the free energies.

For each ligand studied, the magnitude of the total interaction energy with the base form of the receptor was greater than that with the acid form. In each case, the interaction energy with the base form was significantly different than that with the acid form (pair-wise correlation; \( p < 0.001 \)). Thus, we characterize the binding to the acid form as low affinity and the binding to the base form as high affinity.

When the total interaction energy (\( \Delta E_{TOT} \)) is partitioned into Lennard–Jones (\( \Delta E_{LJ} \)) and electrostatic (\( \Delta E_{EL} \)) terms, the comparison between acid and base forms can be described more explicitly. Regardless of the receptor form, the Lennard–Jones interaction has a greater magnitude than the electrostatic interaction. The magnitude of \( \Delta E_{LJ} \) is greater in the acid form than in the base form. The magnitude of \( \Delta E_{EL} \) is greater in the base form than in the acid form. The bias toward the base form in the electrostatic contribution outweighs the bias toward the acid form in the Lennard–Jones contribution. Therefore, as we have already noted, the magnitude of the total interaction energy is greater in the base form.

The \( \Delta E_{LJ} \) interactions are significantly correlated with the experimental affinities, \( -\log K_A \) [15,16], for both acid and base forms (acid: \( r = -0.91, p < 0.001 \); base: \( r = -0.91, p < 0.001 \)). In contrast, the \( \Delta E_{EL} \) interactions are not significantly correlated with the experimental affinities (acid: \( r = 0.60, p > 0.05 \); base: \( r = 0.50, p > 0.10 \)). Thus, we note that the Lennard–Jones interaction is the more important determinant of ligand affinity.

We have also examined the relationships between the two parts of the interaction energy and the RIE of the ligands. The \( \Delta E_{EL} \) interactions are significantly correlated with RIE for both acid and base forms (acid: \( r = -0.84, p < 0.005 \); base: \( r = -0.91, p < 0.001 \)). There is no significant correlation between the \( \Delta E_{LJ} \) interactions and the RIE. Thus,
Table 4
Differences in electrostatic interaction energies, $\Delta \Delta E^{\text{EL}}$, of agonists at the 5-HT$_{3A}$ receptor

<table>
<thead>
<tr>
<th>Primary amino side chains</th>
<th>Tertiary amino side chains</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDOX (RIE = 1.21); -2.295 ± 0.024</td>
<td>MDDM (RIE = 0.07); -2.128 ± 0.041$^b$</td>
</tr>
<tr>
<td>5-HT (RIE = 1.00); -2.241 ± 0.008</td>
<td>BUFO (RIE = 0.20); -2.034 ± 0.025$^b$</td>
</tr>
<tr>
<td>4-HT (RIE = 0.81); -2.164 ± 0.026</td>
<td>4HD (RIE = 0.08); -2.008 ± 0.031$^a$</td>
</tr>
<tr>
<td>TRYP (RIE = 0.48); -2.100 ± 0.023</td>
<td>TRDM (RIE = 0.16); -2.007 ± 0.040$^a$</td>
</tr>
<tr>
<td>5-MOT (RIE = 0.86); -2.117 ± 0.024</td>
<td></td>
</tr>
</tbody>
</table>

Antagonists
- Spiperone: -1.660 ± 0.033
- Ketanserin: -0.224 ± 0.048

*Energies in kcal mol$^{-1}$ ± SEM.
$^a$Values of $\Delta \Delta E^{\text{EL}}$ for this pair of agonists are significantly different; $p < 0.05$.
$^b$Values of $\Delta \Delta E^{\text{EL}}$ for this pair of agonists are not significantly different; $p > 0.05$.

we see that the electrostatic interaction is the more important determinant of ligand efficacy.

A comparison of the differences between the interaction energies ($\Delta E^{\text{BASE}} - \Delta E^{\text{ACID}}$) gives information about the essential basis of efficacy. We obtain the following correlations vs RIE: ($\Delta \Delta E^{\text{TOT}}$, $r = -0.79$, $p < 0.02$; $\Delta \Delta E^{\text{UJ}}$, $r = -0.42$, $p > 0.2$; $\Delta \Delta E^{\text{EL}}$, $r = -0.87$, $p < 0.005$). The striking correlation between $\Delta \Delta E^{\text{EL}}$ and RIE (see Table 4) gives rise to the strong correlation between $\Delta \Delta E^{\text{TOT}}$ and RIE. In addition, the magnitude of $\Delta \Delta E^{\text{EL}}$ is greater than that of $\Delta \Delta E^{\text{UJ}}$, which demonstrates the dominance of the electrostatic contribution to $\Delta \Delta E^{\text{TOT}}$.

For each of the nine agonists studied, marked changes occurred in the ligand–receptor interaction energy when the cysteine was changed between the acid and base forms. In addition, of the five residues (Leu$^3$, Cys$^4$, Trp$^7$, Ile$^8$ and Asp$^{11}$) which make the largest contributions to the interaction energy, the cysteine residue gave the greatest contribution to $\Delta \Delta E^{\text{EL}}$ (see Fig. 5). A plot of the calculated values of $\Delta \Delta E^{\text{EL}}$ for nine ligands versus RIE is shown in Fig. 6. There is a close correlation between the differences in the electrostatic interaction energies and the experimental values of RIE. This supports the model used to analyze the pH-dependence of binding and shows that the electrostatic interactions account for the experimental trends.

For the four agonists included in the pH-dependent binding data, the rank order of the values of $\Delta \Delta G^\circ$ (see Table 1) corresponds to the rank order of the values of $\Delta \Delta E^{\text{EL}}$ (see Table 4). This lends support to the use of the electrostatic interaction energy as a representation of the Gibbs free energy.

We have analyzed the contributions of the residues Cys and Asp to $\Delta \Delta E^{\text{EL}}$. The sum of $\Delta \Delta E^{\text{EL}}$ for Cys and $\Delta \Delta E^{\text{EL}}$ for Asp is highly correlated with RIE ($r = -0.83$; $p < 0.01$). Since this correlation coefficient is close to that for the full receptor model, it may be seen that these two residues make the predominant contributions to ligand efficacy. The electrostatic interaction associated with the change of the Cys from acid to base form produces a small displacement of the ligand position toward the Cys and away from the Asp. The interaction of the ligand with Cys gets stronger and the interaction with Asp gets weaker. As a result, the value of $\Delta \Delta E^{\text{EL}}$ for Cys is negative and the value of $\Delta \Delta E^{\text{EL}}$ for Asp is positive.

3.6. Effect of the structure of a ligand on its efficacy

With respect to the electrostatic interaction of a tryptamine analog with cysteine, the indole ring and the amino side chain make the most important contributions to $\Delta \Delta E^{\text{EL}}$. The indole ring is polar with the positive side, where the nitrogen atom is located, facing the cysteine (see Fig. 1). The side chain contains a high density of positive charge.

Each of the agonist pairs, (MDOX/MDDM), (5-HT/BUFO), (4-HT/4HD) and (TRYP/TRDM), has identical ring structures, but different side chains.
Fig. 5. Average interaction energies for nine tryptamine analogs at the 5-HT₂A receptor model with the five residues which give the largest contributions to the total interaction energy. 'A' designates the acid form of the receptor; 'B' designates the base form of the receptor. 'LJ' designates the Leonard–Jones interaction energy; 'EL' designates the electrostatic interaction energy. Residues are numbered as in Fig. 3. Error bars indicate SEM. The largest difference between the interaction energies for acid and base corresponds to the electrostatic interactions for residue Cys⁴.

For each pair, the first ligand contains a primary amino side chain, whereas the second ligand contains a tertiary amino side chain (see Fig. 4). For each pair, the first ligand has a greater experimental efficacy than the second ligand. In each case, the ligand with the primary side chain has a larger magnitude for ∆ΔE⁺ (see Table 4). The values of ∆ΔE⁺ are significantly different for three of the pairs (MDOX/MDDM, 5-HT/BUFO and 4-HT/4HDM). We see that for a given pair, only the structure of the side chain distinguishes between the efficacies. The explanation of the difference lies in the fact that substitution of

Fig. 6. Plot of ∆ΔE⁺ (difference between electrostatic interaction energies in base and acid forms) versus relative intrinsic efficacy (RIE) for the nine tryptamine analogs in the 5-HT₂A receptor model. Regression equation: ∆ΔE⁺ = −2.014–0.199 RIE (r = −0.87; p < 0.005). Error bars indicate SEM.
two methyl groups spreads the positive charge of the molecule over a larger volume, thus weakening the electrostatic interaction with the cysteine. The spread of the charge over the side chain also affects the interaction of the ligand with the Asp. As stated previously, the change from acid to base form causes the ligand to move away from the Asp, thus making the interaction between the ligand and the Asp weaker. For the ligands with tertiary side chains, this weakening is more pronounced. Therefore, the change in the charge of the cysteine is accompanied by reduced magnitudes for $\Delta \Delta E_{EL}$ and ligand efficacy.

We now consider the structural and electrostatic properties of two ligands, spiperone and ketanserin, which are known to be antagonists at the 5-HT$_{2A}$ receptor. For each antagonist, the magnitude of $\Delta \Delta E_{EL}$ is significantly smaller than the corresponding value for each of the nine agonists already discussed (t-test, $p < 0.001$) (see Table 4). These small differences in electrostatic interaction energy account for the experimental observations that these ligands are antagonists. If, as previously discussed, the electrostatic energy makes the dominant contribution to $\Delta \Delta G^o$, then these small differences indicate that the magnitudes of $\Delta \Delta G^o$ are small. In other words, the two antagonists would be expected to have similar affinities for the acid and base forms of the receptor. This is evidenced by pH-dependent binding experiments [7] which give shallow slopes, relative to those for agonists [see Fig. 2(A)], for plots of log $IC_{50}$ versus pH.

We can account for the small electrostatic energy differences found for the antagonists in two ways. First, the large size of an antagonist molecule spreads its positive charge over a volume that is even greater than in the case of bufotenin. Second, the size and shape of an antagonist molecule is such that attempts to dock the molecule in the same binding site as was used for the smaller agonists place the side chain nitrogen at a greater distance from the sulfur atom of the cysteine. The average N–S distances (in Å) for the agonists 5-HT and BUFO are 8.95 and 8.91, respectively. In comparison, the average N–S distances for the antagonists spiperone and ketanserin are 9.28 and 11.85 Å, respectively. Both of these factors tend to weaken the interaction of the antagonist with the cysteine residue and, in turn, reduce the magnitudes of $\Delta \Delta E_{EL}$.

As the magnitudes of $\Delta \Delta E_{EL}$ for a set of ligands decrease, there is a steady change from high-efficacy agonist to low-efficacy agonist to antagonist. The electrostatic interactions of the ligand with the acid and base forms of the receptor are the distinguishing factors.

3.7. Ramifications of cysteine modulation

The two-state acid–base model which we have described has several features which are parallel to the allosteric ternary complex model [5,6] involving the two receptor states which were designated as $R$ and $R^*$. Within the framework of the acid–base model, agonists display a higher affinity for the base form of the receptor compared with the acid form. In the context of the allosteric model, agonists show a higher affinity for the $R^*$ state compared with the $R$ state. For each case, the difference in affinity is correlated with the efficacy (or intrinsic activity) of the agonist. Mutations which give rise to constitutive activity may act by stabilizing the base form of cysteine. From consideration of the pH-dependence of binding and response, one tends to conclude that the base form of cysteine, $S^*$, is an essential part of the active state of the receptor $R^*$ [5,6].

The electrostatic perturbation of cysteine when it interacts with an agonist is likely to increase the probability of coupling between the receptor and the G protein. The charged (base) form of cysteine may induce changes in other charged or polarizable residues in the receptor. Preliminary calculations indicate that cysteine can make electrostatic interactions with the Asp and Arg, which are parts of the DRY motif, in the intracellular portion of the third transmembrane domain. In turn, these perturbations may give rise to coupling interactions between the receptor and the G protein. Using site-directed mutagenesis experiments [42,43,48–51], several groups have shown the importance of the Asp (or the Glu [51] in the rhodopsin receptor) and the Arg with respect to activation of G protein-coupled receptors.

In the β-adrenergic receptor, experiments have shown that changes in the fluorescence of a probe are related to the nature of the ligand which binds to the receptor [52]. Agonists quench the fluorescence in a manner which is correlated with their efficacies. We understand that these observations can be explained
by the previously described two-state acid–base model involving the modulating cysteine. When the cysteine residue switches to the charged form under the influence of the agonist, the polarity of the region in the neighborhood of the fluorescent probe would tend to increase. An agonist with high efficacy has a strong tendency to shift the receptor equilibrium toward the base form. Therefore, the polarity of the region near the probe increases and the extent of fluorescence decreases.

4. Conclusions

We have shown that the efficacy of a ligand is directly related to the electrostatic perturbation it induces in the receptor. This follows from the close correlation between the observed pH dependence of agonist binding and ligand efficacy. We believe that the agonist-induced shift from the acid to the base form of a conserved cysteine produces the initial change which promotes coupling to the G protein. It becomes clear that only by assuming that the cysteine is a free sulfhydryl can we account for the observed pH dependence. By using a two-state acid–base model, we have derived a set of physical parameters to characterize ligand binding and efficacy for a number of receptors. We believe that this approach can be applied to all G protein-coupled receptors. It is our hope that this work will promote interest in the pH dependence of receptor response and its connection to ligand efficacy.

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References