Acetylcholine and Epibatidine Binding to Muscle Acetylcholine Receptors Distinguish between Concerted and Uncoupled Models*

Richard J. Prince and Steven M. Sine‡§

From the Physiology, Pharmacology and Toxicology Division, School of Biological Sciences, University of Manchester, Manchester M13 9PT, Great Britain and the ‡Receptor Biology Laboratory, Department of Physiology and Biophysics, Mayo Foundation, Rochester, Minnesota 55905

The muscle acetylcholine receptor (AChR) has served as a prototype for understanding allosteric mechanisms of neurotransmitter-gated ion channels. The phenomenon of cooperative agonist binding is described by the model of Monod et al. (Monod, J., Wyman, J., and Changeux, J. P. (1965) J. Mol. Biol. 12, 88–118; MWC model), which requires concerted switching of the two binding sites between low and high affinity states. The present study examines binding of acetylcholine (ACh) and epibatidine, agonists with opposite selectivity for the two binding sites of mouse muscle AChRs. We expressed either fetal or adult AChRs in 293 HEK cells and measured agonist binding by competition against the initial rate of 125I-α-bungarotoxin binding. We fit predictions of the MWC model to epibatidine and ACh binding data simultaneously, taking as constants previously determined parameters for agonist binding and channel gating steps, and varying the agonist-independent parameters. We find that the MWC model describes the apparent dissociation constants for both agonists but predicts Hill coefficients that are far too steep. An Uncoupled model, which relaxes the requirement of concerted state transitions, accurately describes binding of both ACh and epibatidine and provides parameters for agonist-independent steps consistent with known aspects of AChR function.

Acetylcholine receptors (AChRs) from vertebrate skeletal muscle are pentamers of homologous subunits that associate tightly to form a ring around a central channel (1, 2). By initiating global conformational changes throughout the pentamer, ACh opens the channel following brief occupancy and desensitizes following prolonged occupancy. Most mechanistic descriptions of AChR function are founded in the seminal work by Katz and Thesleff (3) more than 40 years ago. They proposed that the AChR spontaneously switches between a low agonist affinity activatable state and a high agonist affinity desensitized state. Furthermore, they proposed that these states are linked in a cyclic rather than a sequential reaction mechanism. To accommodate the stoichiometry of two binding sites per AChR and positive cooperativity in agonist binding, current mechanistic descriptions incorporate some form of the allosteric model of Monod, Wyman, and Changeux (MWC model; Ref. 5). The MWC model accounts for cooperative agonist binding by requiring concerted switching of the two binding sites between low and high affinity states. In addition to accounting for cooperative binding, the MWC model accounts for channel opening or desensitization in the absence of agonist and for nonidentical agonist binding and state functions (5–9).

AChRs from fetal muscle have the subunit composition αβγδ, whereas those from the adult have the composition αββδ. The agonist binding domains are formed at subunit interfaces by αδ and either αγ or αε subunit pairs. Agonists and antagonists select between these three types of binding sites with rank order affinities that depend on both the ligand and the subunit subtype or species (4). The original form of the MWC model postulated identical affinities of the binding sites in a given functional state. However, in the case of the AChR, single channel kinetic analysis showed that in the resting state, the sites can be either indistinguishable or as different as 100-fold in their affinities for agonist, depending on receptor species and subtype (10–12). Nevertheless, nonidentical binding sites are readily incorporated into the MWC model and still allow positive cooperativity in agonist binding.

We recently showed that the novel agonist epibatidine binds to fetal and adult muscle AChRs with unique site- and state-selectivity (13, 14). For receptors in the resting state, epibatidine binds with opposite site-selectivity compared with ACh, showing the rank order of affinity, αγ > αε > αδ. For receptors in the desensitized state, epibatidine selects strongly between the sites, again with the rank order αγ > αε > αδ, unlike ACh, which shows little or no selectivity for this state. Finally, binding measurements at equilibrium reveal Hill coefficients of 0.7–0.8 for epibatidine but values of 1.3–1.4 for ACh.

Here we take advantage of the very different site- and state-selectivities of epibatidine and ACh to quantitatively examine three different models describing receptor activation and desensitization. We fit predictions of each model simultaneously to measurements of ACh and epibatidine binding at equilibrium. Previously measured parameters for the epibatidine- and ACh-dependent steps are taken as constants, whereas the agonist-independent steps are free parameters and assumed to be the same for both agonists. Whereas all three models fit the midpoints of the binding profiles, the MWC model predicts far too steep a dependence on agonist concentration. Accurate fits and consistency with known parameters of receptor function are obtained with an Uncoupled model, which allows the binding sites to switch between functional states independently of each other.

EXPERIMENTAL PROCEDURES

Materials—125I-α-bungarotoxin was obtained from NEN Life Science Products. Epibatidine and proadifen were purchased from Research Biochemicals Inc. The 293 human embryonic kidney cell line (293 HEK)
To account for the difference in membrane potential between the binding experiments in this study (0 mV) and the electrophysiological experiments from which activation parameters were derived (–70 to –100 mV), the channel opening equilibrium constant, Θ, was scaled e-fold per 40 mV. All other parameters were assumed to be independent of membrane potential. \( K_1, K_2, K_{11}, \) and \( K_{22} \) are dissociation constants and are given in \( \mu M \). A is the concentration of agonist. Note that the equations shown here assume random association of agonist with the receptor binding sites.

### Table I

Parameters and equations for agonist binding schemes

| Parameter | Fetal | Reference | Adult | Reference |
|-----------|-------|-----------|-------|-----------|
| ACh       | \( K_1 \) | 21 | 12 | 170 | 11 |
|           | \( K_2 \) | 675 | 12 | 170 | 11 |
|           | \( \Theta \) | 26.3 | 12 | 5.22 | 11 |
|           | \( K_{11} \) | 0.017 | 9 | 0.04 | 11 |
| Epibatidine | \( K_1 \) | 0.017 | 9 | 0.04 | 11 |
|           | \( K_2 \) | 2.0 | 13 | 11.8 | 13 |
|           | \( \Theta \) | 0.9 | 13 | 0.42 | 13 |
|           | \( K_{11} \) | 0.00056 | 14 | 0.00024 | 14 |
|           | \( K_{22} \) | 0.151 | 14 | 0.609 | 14 |

*Scheme 1*

Unliganded = 1 + \( M_p \)

Monoliganded = \( A \{1/\( K_1 \) + 1/\( K_2 \) + \( M_p/\( K_{11} \) + \( M_p/\( K_{22} \) \}) \}

Diliganded = \( A \{ 1 + \Theta \}\{1/\( K_1 \)K_2\} + \( M_p/\( K_{11}K_{22} \) \}

*Scheme 2*

Unliganded = 1 + 2\( M_p \) + \( M_p \)^2

Monoliganded = \( A \{1 + 2M_pK_1 + 1/K_2 + \( M_p/\( K_{11} \) + \( M_p/\( K_{22} \) \}) \}

Diliganded = \( A \{1 + \Theta \}\{1/\( K_1 \)K_2\} + \( M_p/\( K_{11}K_{22} \) \}

*Scheme 3*

Unliganded = 1 + \( M_p \) + \( M_p ' \)

Monoliganded = \( A \{1 + \Theta \}\{1/\( K_1 \)K_2\} + \( M_p/\( K_{11} \) + \( M_p ' /\( K_{22} \) \}

Diliganded = \( A \{1 + \Theta \}\{1/\( K_1 \)K_2\} + \( M_p/\( K_{11}K_{22} \) \}

*Scheme 4*

Unliganded = 1 + \( M_p \)

Monoliganded = \( A \{1/\( K_1 \) + 1/\( K_2 \) \}

Fractional occupancy = (monoliganded/2 + diligated)/unliganded + monoliganded + diligated)

where \( K_1 \) and \( K_2 \) are dissociation constants for binding to sites in the resting state \((R)\), \( K_{11} \) and \( K_{22} \) are dissociation constants for binding to sites in the desensitized state \((D)\), \( A \) is agonist, \( O \) is the open state, \( \Theta \) is the channel opening equilibrium constant, and \( M_0 \) is the allosteric constant governing desensitization. The \( D \) state is assumed to correspond to the high affinity state induced by exposure to local anesthetics such as proadifen (8, 17). Although in its original form, the MWC model accounts for positive cooperativity in agonist binding, apparent negative cooperativity can arise if the two sites differ in their affinities for agonist and \( M_0 \) approaches limiting small or large values. Except for the allosteric constant \( M_0 \), all parameters in Scheme 1 have been determined by single channel kinetic analysis or by measurements of agonist binding to desensitized receptors (Table I). Because the MWC model does not take into account the equilibrium between unliganded states, it is important to regard it as an intrinsic property of the receptor and should be agonist-independent. We therefore fit Scheme 1 to measurements of epibatidine and ACh binding simultaneously, allowing only \( M_0 \) to vary (Table II). The MWC model fits the midpoints of the binding curves but predicts Hill coefficients significantly steeper than observed experimentally.

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was obtained from the American Type Culture Collection. Mouse AChR subunit cDNAs were provided by Drs. Norman Davidson, Paul Gardner, and John Morlie.

**Ligand Binding Measurements—**Receptors were transiently expressed in 293 HEK cells as described (15, 16). Two or three days after transfection, the growth medium was removed and the cells were rinsed with phosphate buffered saline and incubated with phosphate-buffered saline containing 1 \( \mu M \) diisopropylphosphofluoridate for 15 min. Cells were centrifuged at 1000 \( \times g \) for 1 min and resuspended in potassium Ringer’s solution (140 mM KC1, 5.4 mM NaCl, 1.8 mM CaCl\(_2\), 1.7 mM MgCl\(_2\), 25 mM HEPES, 30 mg/liter bovine serum albumin, and adjusted to pH 7.4 with 10–11 mM NaOH). Agonist occupancy was determined by competition against the initial rate of \(^{125}\text{I}-\text{bungarotoxin binding as described (16).} In brief, cells expressing AChRs were equilibrated with agonist for 40 min prior to the addition of 5 mM \(^{125}\text{I}-\text{bungarotoxin. The cells were then incubated for a further 20–40 min to allow occupancy of at most 50% of the binding sites by \(^{125}\text{I}-\text{bungarotoxin. The total number of sites was determined by incubating with 25 mM \(^{125}\text{I}-\text{bungarotoxin for 20–40 min. Nonspecific binding was determined in the presence of 10 mM carbachol. The cells were harvested using a Brandel Cell Harvester and counted in a y-counter.**

**Data Analysis—**Equations in Table I were fitted to the data using MLAB (Civilized Software Inc), and the results were plotted using Prism (GraphPad Software). Note that for simplicity, Schemes 1–3 show sequential binding of agonist but that the equations used in curve fitting describe random binding to the two agonist binding sites.

**RESULTS**

The **MWC Model—**Epibatidine and ACh bind to mouse AChRs with similar apparent dissociation constants under equilibrium conditions (Fig. 1). However, the two agonists differ strikingly in their degree of cooperativity in binding; for both fetal and adult AChRs, epibatidine binds with Hill coefficients of 0.7–0.8, whereas ACh binds with Hill coefficients of 1.3–1.4 (Table III). The following MWC-based model has been applied to the AChR to describe cooperative binding of agonist to an equilibrium population of receptors in the resting, open channel and desensitized states (8, 9),

![Scheme 1](http://www.jbc.org/Downloadedfrom)
Separate fitting of the MWC model to either the epibatidine or ACh data produced similar results, suggesting that the divergence between theory and experiment is not an artifact of simultaneous fitting.

The Uncoupled Model—The MWC model predicts positive cooperativity in agonist binding because sites on the same receptor are constrained to be in the same functional state. To better describe the binding profiles for ACh and epibatidine, we relaxed the constraint of functional symmetry to achieve greater heterogeneity of the binding species. The following Uncoupled model (Scheme 2) allows independent switching of the two binding sites from one state to the other, and variable coupling between the sites (18).

Unlike the MWC model, the Uncoupled model allows binding sites of an individual receptor to switch between functional states in the absence of agonist. The allosteric constant $M_1$ describes the equilibrium between $R$ and $D$ states for the binding site that first enters the desensitized state, whereas $M_2$ describes the equilibrium for the second site. Receptors with one site in the $D$ state are assumed to be desensitized, so that either of the binding sites can initiate desensitization. The remaining agonist-dependent parameters are the same as described for Scheme 1.

Simultaneous fits of the Uncoupled model to measurements of ACh and epibatidine binding accurately describe the binding profiles for both fetal and adult receptors (Fig. 1B). In particular the degree of cooperativity for both agonists is fully accounted for (Table III). The fitted value of $M_1$ is roughly 10-fold smaller than $M_2$, allowing just enough site heterogeneity to broaden the binding profiles for both agonists yet still accounting for positive cooperativity in ACh binding (Table II). Separate fitting to the epibatidine data yielded $M_1$ and $M_2$ values that accurately described the data for ACh, whereas separate

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**Fig. 1.** Equilibrium binding of ACh and epibatidine to fetal and adult acetylcholine receptors. The MWC model (panel A) and Uncoupled model (panel B) were fit to ACh and epibatidine (Epi) data simultaneously, allowing only the allosteric constants $M_0$, $M_1$, and $M_2$ to vary. The fitted values of $M_0$, $M_1$, and $M_2$ are given in Table II. All other parameters were fixed to the values in Table I. Data are means of three to five experiments. The error bars represent the S.E.

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**Scheme 2.** Agonist Binding to Nicotinic Receptors
fitting to the ACh data yielded $M_1$ and $M_2$ values that failed to describe the epibatidine data (not shown). Simultaneous fitting therefore provides meaningful results for both ACh and epibatidine binding. Thus the Uncoupled model, together with known parameters for agonist binding and channel gating, accounts for all aspects of equilibrium binding for ACh and epibatidine.

**Two Desensitized State Model**—Since the work of Katz and Thesleff (3), considerable evidence has accumulated to show that desensitization is in fact a heterogeneous phenomenon. Time constants for desensitization have been reported on time scales ranging from milliseconds to minutes, leading to the consensus that there are at least two predominant desensitized states; one has fast onset and fast recovery, whereas the other has slow onset and slow recovery (19–21). We therefore tested a model that incorporates two distinct desensitized states.

One of the desensitized states ($D$) is assumed to correspond to desensitization with slow onset and slow recovery and is the high affinity state induced by prolonged exposure to agonist or by local anesthetics (8,17). The allosteric constant $K_D$ describes the equilibrium between $D$ and $M$ states and is analogous to that in the MWC model. The second desensitized state ($I$) corresponds to desensitization with rapid onset and rapid recovery, binds agonist with dissociation constants $K_{D1}'$ and $K_{D2}'$, and equilibrates with the $R$ state according to the allosteric constant $K_{I}'$.

Simultaneous fitting of Scheme 3 to binding data for epibatidine and ACh requires six free parameters: $M_0$, $M_0'$, $K_{D1}'$ and $K_{D2}'$ for epibatidine, and $K_{D1}'$ and $K_{D2}'$ for ACh. The remaining parameters are known and were constrained to the values used in fitting the MWC and Uncoupled models (Table I). We found that simultaneous fits of Scheme 3 described the data approximately as well as the Uncoupled model (not shown) but yielded estimates of the six free parameters that were highly independent (dependence values close to 1 for all parameters), suggesting that a unique solution cannot be found.

Further evaluation of Scheme 3 requires knowing one or more of the six free parameters or constraining them in a reasonable way. We have no direct information on $K_{D1}'$ and $K_{D2}'$ for either agonist for fetal or adult AChRs. However, for ACh binding to the adult mouse AChR, we know that $K_1$ and $K_2$ for the $R$ state are indistinguishable, as are $K_{D1}$ and $K_{D2}$ for the $D$ state (Table I). Thus it seems reasonable to assume that $K_{D1}'$ and $K_{D2}'$ for the $I$ state are identical and to assign a single value $K_{I}'$. Furthermore, we can constrain the fraction of receptors desensitized in the absence of agonist to a conservative value of 5%, or $M_0 + M_0' = 0.05$. Thus, we fit Scheme 3 to the ACh data by assigning $K_{I}'$ to various values, varying $M_0$ and $M_0'$, but constraining $M_0 + M_0' = 0.05$. The fit accurately described ACh binding to the adult AChR, with a $K_{I}'$ of 0.32 μM, $M_0 = 0.0$ and $M_0' = 0.05$. However, the value of $M_0$ was vanishingly small, predicting little or no receptor in the $D$ state and that the $I$ state predominates over the $D$ state under all conditions. Both of these predictions conflict with the observation that the $D$ state predominates over the $I$ state in single channel recordings (28,29). Thus the two desensitized state model, although able to describe our data, fails to account for known aspects of AChR function.

**Selective Protection with α-Conotoxin M1**—We sought to further distinguish the MWC and Uncoupled models by preventing access of agonist to one of the two binding sites. We reasoned that if one site could be selectively occupied by an antagonist, or effectively removed from the $R$ to $D$ equilibrium, both models simplify to the following one-site scheme.

In Scheme 4, $X_a R$ and $X_a D$ represent receptors in the resting and desensitized states, respectively, which have one site occupied by the selective antagonist. $K$ and $K_D$ are dissociation constants for agonist binding to the resting and desensitized states, respectively, but correspond to the remaining site accessible to agonist. $M_r$ is the allosteric constant governing the equilibrium between $R$ and $D$ states and is analogous to the allosteric constants in the MWC and Uncoupled models.
selectively occupy one of the two binding sites, we used α-conotoxin MI (CTx), a peptide toxin from predatory marine snails that binds to the αδ site with nanomolar affinity, to the αε site with micromolar affinity, and to the αγ site with tens of micromolar affinity (Ref. 22; Table IV). Recent studies showed that CTx dissociates very slowly from the αδ site, based on the stability of αδ-CTx complexes during sucrose gradient sedimentation (23). Following incubation and removal of CTx, measurements of $^{125}$I-α-bungarotoxin binding show that 50% of either fetal or adult AChR binding sites are occupied by CTx, a percentage that remains constant more than 6 h following removal of the free CTx (Fig. 2). Thus, CTx selectively and irreversibly occupies the αδ site.

Following selective occupancy by CTx, receptors were equilibrated with specified concentrations of either ACh or epibatidine, and fractional occupancy was measured. For ACh, apparent affinity decreases nearly 10-fold compared with the unprotected fetal or adult receptor controls, and the binding profiles broaden to approach Hill coefficients of one, as expected for binding to a single site and low affinity of free αγ (fetal AChR) or αγ (fetal AChR) sites (Fig. 3; Table V). Analogously, for epibatidine, apparent affinity increases and the binding profiles sharpen to approach Hill coefficients of one, as expected for binding to a single site, and high affinity of free αε or αγ sites. Thus, following selective occupancy by CTx, the ACh and epibatidine binding profiles shift in opposite directions, as expected from their opposite site-selectivity.

To estimate the allosteric constant $M_C$, we fit Scheme 4 simultaneously to the ACh and epibatidine data. We constrained the dissociation constants for binding to resting and desensitized states to known values, and varied $M_C$. For ACh binding to αγ and αε sites in the resting state, we assigned $K_1$ to the low affinity values of 675 and 170 μM, respectively (Table I). Conversely, for epibatidine binding to αγ and αε sites in the resting state, we assigned $K_1$ to the high affinity values of 2 μM and 118 μM, respectively. Similarly, for ACh binding to αγ and αε sites in the desensitized state, we assigned $K_2$ to the values of 17 and 40 nM, respectively (Table I). For epibatidine binding to αγ and αε sites in the desensitized state, we assigned $K_2$ to the high affinity values of 0.56 and 2.4 nM, respectively. The resulting fitted curves accurately describe both ACh and epibatidine binding to selectively protected fetal and adult AChRs (Fig. 3). Moreover, the fitted $M_C$ values of 0.0016 (fetal) and 0.004 (adult), are within 2-fold of $M_1$ derived from fits of the Uncoupled model to unprotected, control data, but are more than 5-fold greater than $M_C$ derived from the MWC model (Table II). Thus the Uncoupled model accurately describes equilibrium agonist occupancy of one binding site in the absence of the influence of the second site.

To be sure that CTx MI itself does not perturb the equilibrium between resting and desensitized states, we measured CTx MI binding in the presence and absence of the desensitizing agent proadifen. If CTx desensitizes the receptor, its affinity should increase in the presence of proadifen. Incubation with proadifen does not affect CTx affinity for the adult receptor and slightly reduces affinity for fetal receptors (Table IV).
Thus, CTx does not disturb the equilibrium between resting and desensitized states of the receptor.

**DISCUSSION**

For many years state transitions of the muscle AChR have served as prototypes for understanding function of the superfamily of neurotransmitter-gated ion channels. The AChR can exist in at least three functional states: resting, open, and desensitized (6), and various models have been proposed which link these states. However, to describe agonist binding at equilibrium, the interdependence of parameters in the models requires that some of them be measured directly to provide adequate constraints for fitting. Recent advances in single channel kinetic analysis provide direct measurement of agonist dissociation constants for binding to the resting state of the receptor, as well as the equilibrium constants that govern channel gating (10–13). The present study uses these kinetically determined parameters to constrain fits of various models to measurements of ACh and epibatidine binding at equilibrium. Our data are accurately described by an Uncoupled model, which allows the binding sites to switch between functional states independently of each other.

Applied to the AChR, the MWC model proposes that receptors exist in three main states: resting, open, and desensitized. It further proposes that both ACh binding sites change their conformational states simultaneously. Within the framework of the MWC model, the desensitized state is less stable than the resting state in the absence of agonist but is more stable when agonist is bound. In general, the MWC model accounts for positive but not negative cooperativity in agonist binding. In fitting the MWC model to epibatidine and ACh binding data, the sole unknown parameter is the allosteric constant $M_0$. Changes in $M_0$ not only shift the binding curve along the agonist concentration axis, but they also change its slope (24). We find that the MWC model fits the midpoints of binding curves for both epibatidine and ACh. However, for both agonists the predicted binding curves are far too steep, leading us to consider alternatives to the MWC model.

The Uncoupled model was originally developed to explain apparent negative cooperativity in agonist binding to subunit-omitted, pentameric receptors of the form $\alpha_2\beta_2$ or $\alpha_2\gamma_2$ (18). It expands the MWC model by allowing independent isomerization of the two binding sites between resting and desensitized states. Thus, the Uncoupled model allows individual receptors to have one binding site in the resting state and the other in the desensitized state. In the limit of $M_0 \gg M_1$, the Uncoupled model becomes identical to the MWC model. Although the Uncoupled model is similar in appearance to the model of Koshland et al. (Ref. 25; KNF model), it is distinct in that state transitions are not ligand induced but rather are spontaneous conformational changes stabilized by agonist. Analogous to the Uncoupled model, a “Stepwise” model was proposed to describe receptor activation at the single channel level because of a better statistical description than the standard linear model, which itself is a subset of the MWC model (26).

We find that the Uncoupled model accurately describes both the Hill coefficient and $K_{app}$ for ACh and epibatidine binding at equilibrium. For both fetal and adult receptors, the fitted value of $M_0$ is 10–20-fold greater than $M_1$, indicating that isomerization of one binding site to the desensitized state facilitates isomerization of the second site. Thus, similar to the MWC model, the Uncoupled model accounts for positive cooperativity in agonist binding. However, the Uncoupled model also accounts for apparent negative cooperativity in agonist binding because it allows build up of hybrid resting/desensitized species. Epibatidine unmasks hybrid species because of its 300-fold difference in affinity for the desensitized binding sites, together with its 15- to 75-fold difference in affinity for the resting state sites (13, 14). The overall consequence at equilibrium is negative cooperativity in epibatidine binding, with Hill coefficients less than one.

Unlike agonists, the competitive antagonist CTx MI does not perturb the equilibrium between the resting and desensitized states. Under conditions of selective occupancy by CTxs, both the MWC and Uncoupled models simplify to a system governed by a single allosteric constant $M_C$. Removal of the $\alpha\delta$ binding site from the R to D equilibrium eliminates its free energy contribution to drive desensitization of the unoccupied $\gamma\gamma$ or $\alpha\epsilon$ sites. We find that for both fetal and adult receptors, the value of $M_C$ is very close to the value of $M_1$ derived from fits of the Uncoupled model to control, unprotected AChRs. Thus, protection experiments with CTx provide an independent measurement of $M_1$ and further support the Uncoupled model.

Studies over the last 40 years have shown desensitization to occur on time scales ranging from milliseconds to minutes. Although some of this variability undoubtedly owes to species and methodological differences, considerable evidence has accumulated to show that the receptor desensitizes at multiple rates. At mouse AChRs, brief exposure to agonist using liquid filament switch techniques reveals “fast” desensitization, with onset rates on the order of 20 s$^{-1}$ and recovery rates as fast as 3 s$^{-1}$ (20, 21). In contrast, ion flux studies with the mouse AChR demonstrate much slower desensitization onset and recovery, with rates on the order of 0.01–0.02 s$^{-1}$ (27). Fast and slow desensitization are also observed at the single channel level (28, 29). At high agonist concentrations, activation episodes of single receptors appear in “bursts” of openings separated by closings of around 100 ms duration. These sub-second closings are thought to represent dwellles in the fast desensitized state, followed by return to the resting/activatable state. Bursts of openings, in turn, group into “clusters” separated by closings tens of seconds long. This longer class of closings corresponds to dwellles in the slow desensitized state. Within the framework of the Uncoupled model, multiple rates of desensitization are expected. We hypothesize that “fast” desensitization results from transition from the RR to the hybrid RD state, whereas

### Table V

|          | Adult | Fetal |
|----------|-------|-------|
| $K_{app}$ | $n_H$ | $K_{app}$ | $n_H$ |
| ACh      | 1.5 ± 0.2 | 1.3 ± 0.02 | 1.3 ± 0.2 | 1.4 ± 0.06 |
| Conotoxin labeled | 8.1 ± 0.9 | 0.9 ± 0.1 | 9.1 ± 1.1 | 0.82 ± 0.04 |
| Epibatidine  | Control | 1.2 ± 0.1 | 0.68 ± 0.02 | 0.57 ± 0.06 | 0.76 ± 0.03 |
| Conotoxin labeled | 0.6 ± 0.06 | 0.9 ± 0.02 | 0.32 ± 0.06 | 0.88 ± 0.1 |

**AChRs were pre-incubated for 1 hr with conotoxin M1 (adult AChR, 30 nM; fetal AChR, 1 μM) to selectively occupy the αδ binding site. Agonist binding was then determined as described under “Experimental Procedures.” The parameters are derived from fits of the Hill equation to the data in Figure 3 and are expressed ± S.E. $K_{app}$ is the apparent dissociation constant and $n_H$ is the Hill coefficient.**
“slow” desensitization results from transition from the RD to the DD state.

Previous workers sought to explain multiple rates of desensitization using models that retain strict coupling between the binding sites but incorporate two separate desensitized states differing in their affinity for agonists. One desensitized state (the D state) is assumed to bind agonist with very high affinity compared with the resting R state, and the second desensitized state (the I state) is assumed to bind agonist with affinity intermediate between the R and D states. Stopped-flow studies using Torpedo AChRs and the fluorescent agonist danyl-C6-choline revealed binding to a class of sites with apparent affinity of approximately 1 µM (30). These results were interpreted as evidence for an intermediate affinity, fast desensitized (I) state because the 1 µM apparent dissociation constant was intermediate between the EC50 for receptor activation and the dissociation constant of the high affinity desensitized state. However, more recent stopped flow measurements suggest that the fast and slow desensitized states of the Torpedo receptor have indistinguishable dissociation constants for danyl-C6-choline (31). Further, single channel kinetic analysis shows that the two binding sites of the Torpedo AChR differ substantially in agonist affinity for the resting state (10). The higher affinity site in the resting state, thought to be the αδ site, binds ACh and the agonist NDB-5-acycholine with dissociation constants of ~4 and ~2 µM, respectively (10, 32). Thus, the 1 µM KD I state observed by stopped flow with danyl-C6-choline (30) may correspond to occupancy of the αδ binding site in the resting state. Overall, substantial evidence indicates that the AChR desensitizes at multiple rates, but there is little direct evidence that these desensitized states differ from each other in their affinities for agonists.

Recent studies of other ligand gated channels also reveal allosteric behavior that cannot be accommodated by MWC-based models. Homotetrameric cyclic nucleotide-gated channels can open with 1–4 cGMPs bound and have distinct conductance states associated with each state of agonist occupancy (33). For each conductance state, the probability of opening was not linearly related to ligand occupancy. These findings suggest complex interactions between the subunits, which can be explained by expanding the MWC model to incorporate features of the Uncoupled and KNF models (34). Overall, results from cyclic nucleotide-gated channel and the AChR serve to emphasize that the MWC, KNF, and Uncoupled models are all subsets of a more general description of allosteric interactions (35).

In conclusion, the classic MWC model does not quantitatively describe the binding of ACh and epibatidine to mouse muscle AChRs. The simplest model that describes equilibrium agonist binding is an “Uncoupled” model in which state transitions of the two receptor binding sites are not strictly coupled.

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