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Topical Review

Activation of Skeletal Muscle Nicotinic Acetylcholine Receptors

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I. Introduction

The scientific world was first introduced to the nicotinic receptor by Langley (1906), who reported a "receptive substance . . . combining with nicotine and curare" and associated with the contraction of skeletal muscle. Since then, the nicotinic receptor has been studied extensively, and has served as a model for the rapid gating of ionic permeability changes by a neurotransmitter.

The first intracellular recordings of synaptic transmission demonstrated a localized conductance increase associated with the action of acetylcholine (ACh) (Fatt & Katz, 1951, 1952). Although reversible binding of a drug with its receptor was not a new concept (Clark, 1933), the distinction between the agonist binding reaction and the subsequent opening of an ion channel was not made until later (del Castillo & Katz, 1957*a*,*b*). Virtually all subsequent work on the ACh receptor has attempted to answer two kinds of question that are intrinsic to the del Castillo and Katz proposal. On the one hand, we would like to know something about the rates and affinities of the ACh binding reactions and of the conformational changes. On the other, we would like to learn about structural components of the AChR receptor that are associated with these events. Significant progress has been made in addressing these questions, but they are not as yet fully answered.

At the time of the last review of AChR kinetics in this journal (Adams, 1981), our understanding of the kinetic behavior of currents activated by ACh was based principally on macroscopic measurements of current relaxations and fluctuations. Analysis of fluctuations (noise) in the current recorded from voltage-clamped frog muscle fibers in the presence of low concentrations of ACh indicated a single spectral component (Lorentzian), which could be accounted for if ACh induced rectangular single channel openings with a mean duration of 1 msec. According to the prevailing view at that time, receptor activation occurs in two distinct phases in which the fast binding of agonist molecules is followed by relatively slow isomerization of the channel to the open state (Magleby & Stevens, 1972; Anderson & Stevens, 1973). In retrospect, it is now apparent that, even prior to the use of single channel recording techniques, this view was inconsistent with the then available data. There was good evidence that a high concentration of agonist can open a relatively large (>90%) proportion of available channels. Furthermore, the channel closing rate (the inverse of the open channel lifetime) was assumed to be 1000 sec^{-1}. Together, these require a channel opening rate well in excess of 1000 sec^{-1}, which would make it the same order of magnitude as independent estimates of the agonist dissociation rate.

Colquhoun and Hawkes (1977) extended the treatment of Stevens (1972) by applying the matrix methods of Eigen and de Maeyer (1963) to the behavior of ligand-gated ion channels. Using the evidence from relaxation and fluctuation measurements, they predicted that isomerization of the channel to the open state might be at least as fast as agonist dissociation and that as a consequence a receptor with agonist bound would flicker between open and closed states, producing a series or "burst" of closely spaced openings. They indicated that therefore the 1-msec spectral component seen in fluctuation experiments was an estimate of the mean duration of bursts of channel openings rather than single openings.

Over the last ten years, experimental advances in two areas, the use of high resolution measurements of currents through single ACh-activated ion

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channels and alterations of the structure of subunits comprising the acetylcholine receptor, have held out the hope that it might be possible to understand the kinetic and structural questions outlined above. In this review, we have chosen to focus on three particular questions which have occupied researchers over the last ten years and have depended in large measure on these newer experimental approaches. First, for the major classes of acetylcholine receptor found in muscle, what is now known about their activation properties? Second, how many kinds of ACh receptor are there in muscle membrane? Third, what has been learned about the structural features of the receptor which may be important for understanding the activation of the receptor? There are a number of other important questions about ACh receptor function, including the mechanisms of desensitization and certain types of channel blockade, which remain largely unresolved and require further investigation.

II. Single Channel Currents Reveal Rates for Receptor Activation

This discussion of the activation behavior of neuromuscular ACh receptors will be organized as follows. First, we will present a general overview of the kinds of data used to analyze receptor activation, and how kinetic information can be extracted from these data. Second, the kinds of activation models which have been considered and the implications of those models will be outlined. Finally, some of the experimental observations and how they relate to particular activation schemes will be summarized.

The now classical kinetic model for activation of the neuromuscular ACh receptor is:

$$A + R \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} AR + A \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} A_2R \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} A_2R^*. \qquad (1)$$

In this model, the closed or inactive receptor/channel (R) undergoes the sequential binding of two agonist molecules, with association rates k_{+1} and k_{+2} and dissociation rates k_{-1} and k_{-2} before a conformational change occurs to the open channel state (R^*), with opening rate constant β and closing rate constant α (Katz & Thesleff, 1957). This model is an extension of the single agonist binding model of del Castillo and Katz (1957*a*,*b*), and still summarizes reasonably well both the major features of single channel records and macroscopic aspects of cholinergic function. The critical question that has challenged investigators over the last ten years has been to define the microscopic rate constants (α, β, etc.) that are associated with the above scheme.

A. Bursts and Clusters of Openings

1. Bursts of Openings at Low Agonist Concentrations Reveal Rates for Steps Close to the Open State

Within single channel data, it is the duration and frequency of occurrence of closed periods between openings which can provide the most information about the mechanisms of receptor activation and the associated rate constants. Referring to scheme 1 above, when an open channel closes to the A_2R state, two things may happen. The channel can either lose a bound agonist molecule (returning to the AR state), or it can re-open (A_2R^* state). If β, the channel opening rate is of the same order of magnitude or greater than the agonist dissociation rate k_{-2}, then the single channel current will be interrupted by short channel closures or gaps.

The particular kind of grouping observed at low agonist concentrations is called a "burst" (Colquhoun & Hawkes, 1977, 1981). Colquhoun and Sakmann (1981; *see also* Nelson & Sachs, 1979) first observed bursts of openings, which are now known to be a general feature of single channels activated by ACh.

Based on scheme 1, two kinds of closed gaps may, in fact, be expected to occur within bursts at low agonist concentrations. The first kind of gap (often called the fast gap) predicted at low agonist concentrations reflects the brief closing of the channel to state A_2R. At the low concentration limit, there would be on average β/k_{-2} of these fast gaps within a burst. Furthermore, the mean duration of such closures within bursts would be given by $1/(\beta + k_{-2})$. For simplicity, we shall refer to this particular type of closure as an A_2R closure. The mean burst duration would be $(1 + (\beta/k_{-2})(1/\alpha) + (\beta/k_{-2})(1/(\beta + k_{-2}))$. If the channel opening and agonist dissociation rates are rapid compared to the channel closing rate, the right-hand term can be neglected.

In cases to which scheme 1 applies, therefore, accurate determination of the duration and number of the A_2R closures within bursts and the mean duration of open periods within bursts will define the important kinetic parameters, β, α, and k_{-2}. This is the most generally used strategy for estimating the activation characteristics of ACh receptors.

A second type of closed gap (intermediate gap) within a burst is predicted to occur when a channel closes to the A_2R state, loses one of its ligands (to

return to the AR state), but before it can lose the remaining ligand, binds another ligand to return to the A_2R state, and then re-opens. This sequence of events is relatively improbable at low agonist concentrations, but becomes more likely at higher agonist concentrations. Analysis of single channel records at low agonist concentrations typically reveals a second closed interval component of longer duration and low frequency of occurrence (Sine & Steinbach, 1984*a*; Colquhoun & Sakmann, 1985). However, it has not been established that the experimentally observed gaps are indeed the "intermediate gap" expected from scheme 1.

2. *Clusters of Openings at High Agonist Concentrations Reveal Rates for Agonist Binding*

ACh receptors at the neuromuscular junction exhibit desensitization (Katz & Thesleff, 1957). Continuous exposure to ACh, even at concentrations which produce barely detectable activation of current, results in a reversible decrease in the ability of channels to open in response to ACh. Desensitization is thought to reflect a conformational alteration of the receptor to some set of states from which opening is much less likely. With ACh concentrations above about 20 μM, more than 99% of receptors are desensitized at equilibrium. However, desensitized receptors will be expected to return with some finite probability to the set of activatable states described by scheme 1. When that occurs, a series of openings and closings governed by the reaction rates of scheme 1 will then occur, followed by a long silent period resulting from the return of that channel to a desensitized condition. Such groupings of openings at higher agonist concentrations have been termed "clusters" to distinguish them from the mechanistically different "bursts" observed at lower agonist concentration. Such behavior was first described by Sakmann et al. (1980) in rat muscle.

Although desensitization poses a severe problem for the analysis of the dose-response characteristics of the whole-cell cholinergic current, at the single channel level desensitization is an advantage. Since clusters are believed to reflect the behavior of a single ion channel protein, the analysis of events within a cluster can in theory provide direct information not only about the dose-response behavior of the ACh receptor, but also about the microscopic kinetic transitions among activatable states. In particular, as the agonist concentration is increased the time spent in closed periods during clusters will diminish in accordance with the reaction rates of the activation process. Thus, the fraction of time that a channel is open during a cluster (i.e., the probability that the channel is open or p_{open}) provides a direct indication of the equilibrium distribution among open and closed states as a function of agonist concentration in the absence of desensitization.

Several parameters can be obtained from the evaluation of clusters over a range of agonist concentrations. The concentration of agonist which results in a half-maximal p_{open} is essentially equivalent to a rapid flux measurement of the apparent K_a for 50% activation of receptors, but in the absence of desensitization. At higher agonist concentrations p_{open} approaches an asymptote of $\beta/(\alpha + \beta)$, which is the fraction of doubly liganded channels which are open at saturating concentrations of agonist. Also, at saturating agonist concentrations, the mean duration of the gaps within a cluster approaches the inverse of the channel opening rate, $(1/\beta)$. The duration of this concentration-dependent gap, frequently called the β' gap, at limiting high agonist concentrations can therefore provide an estimate of β independent of estimates obtained at low agonist concentration.

The properties of clusters of openings at high agonist concentrations have been exploited in a number of studies (Ogden & Colquhoun, 1983; Auerbach & Lingle, 1986, 1987; Igusa & Kidokora, 1987; Colquhoun & Ogden, 1988) and, in some cases, information about ACh receptor activation has been inferred from both low and high concentration information. The analysis of receptor activation at concentrations of agonist in an intermediate range (e.g., 1–10 μM ACh) has been harder, since unambiguous identification of clusters in this concentration range can be difficult.

A critical assumption in this type of work is that clusters at high agonist concentration do in fact represent the normal behavior of the receptor as it undergoes transitions among conformational states in the normal activation pathway of the receptor. This assumption is supported by the fact that the properties of the receptor obtained from analysis of clusters generally agree with the properties of the receptor predicted from data obtained at low agonist concentrations (Ogden & Colquhoun, 1983; Colquhoun & Sakmann, 1985; Auerbach & Lingle, 1987; Sine & Steinbach, 1987).

To summarize, both low and high agonist concentrations have been used in evaluating ACh receptor activation. Channel openings activated by cholinergic agonists are organized into different types of groupings depending on whether the data are obtained at low or high agonist concentrations. The mechanistic significance of such groupings, therefore, differs.

To estimate reaction rates, the frequency and/

or duration of particular open and closed intervals are interpreted using equations relating the measured intervals to those rates through procedures defined by Colquhoun and Hawkes (1977, 1981). In addition, estimates of concentration-dependent rates ($k_{+1}A$, $k_{+2}A$) require quantitative dose-response data. Even concentration-independent rates (e.g., β and k_{-2}) must be examined for consistency across a range of concentrations. An approach which may offer some advantages in obtaining good estimates of rate constants would be to fit the raw data or the interval distributions directly with a kinetic model (Horn, 1987; Sine et al., 1990), thereby eliminating the need to estimate interval time constants and amplitudes. A significant extension of this procedure will be the simultaneous fitting of models to data collected over a range of conditions, e.g., voltages and/or concentrations.

B. Several Factors Have Complicated the Assessment of Activation Behavior

As described above, the estimation from single channel data of the reaction rates for activation of the ACh receptor should be straightforward. Unfortunately, several difficulties, both analytical and technical, have conspired to complicate the determination of reaction rates. The main problem is that single channel records usually have more kinds of gaps within bursts than are predicted by scheme 1 (*see below*). In addition, in some cases that have been carefully examined a nominal single component may actually be better described as being composed of two exponentials (Colquhoun & Sakmann, 1985).

A second analytical difficulty is that it has not been easy to quantify the properties of the closed gaps expected to be involved in activation. In some cases, two components may be difficult to separate (Auerbach & Lingle, 1987). In other cases, an expected gap component may contribute a relatively small proportion to the total number of observed gaps. Both of these situations may be entirely consistent with a particular model and set of rate constants (Colquhoun & Sakmann, 1985; Auerbach & Lingle, 1987; Sine & Steinbach, 1987), but when components are poorly resolved in the closed time distributions, estimates of reaction rates will be unreliable.

A related problem, which is unique to high agonist concentration experiments, is that the closed interval distribution may be dominated by brief gaps resulting from blockade of the ion permeation pathway by agonist (Sine & Steinbach, 1984*b*; Ogden & Colquhoun, 1985). Although such blocking gaps may be exceedingly short, their frequent occurrence tends to skew estimates of gating gaps, especially at higher agonist concentrations. The final difficulty in accurately assessing kinetic rates, largely a technical one, is that the shortest duration closed intervals involved in ACh receptor activation are close to the limits of resolution. A large fraction of the shortest events are not detected with single channel recording methods. As a result, estimates of the duration and number of these gaps has to be made following corrections based on the number of gaps thought to be missed, given the particular recording bandwidth. Although a number of procedures for such corrections have been developed (Blatz & Magleby, 1986; Roux & Sauve, 1985; Crouzy & Sigworth, 1990), all are intrinsically model dependent. Furthermore, there is no assurance that the measured fast gaps represent a single population.

In the case of estimates of p_{open}, two types of difficulties arise. First, the problem of channel blockade by agonist can influence p_{open} estimates as saturation of receptor activation is approached. Hence, an estimate of p_{open} without a correction for channel block will give an inaccurate estimate of activation parameters. However, since acetylcholine is much more potent as an agonist than a channel blocker, the concentration at which half-maximal activation occurs can be estimated without too much error. The second problem with p_{open} estimation is that some gaps, which occur within clusters, may not be involved in the channel activation pathway (*see below*). If such gaps not are excluded, they will result in an underestimate of p_{open}. Although in some cases these gaps do not occur at a rate high enough to significantly alter p_{open}, it is better to estimate the number and duration of such gaps and simply correct the p_{open} estimate. Of course, this does not address the question of what physical process such gaps actually represent.

These problems have resulted in some disarray in the assessment of activation rates for the AChR. Different research groups have associated different closed time components with activation events to make kinetic estimates. In addition, the measurement of the duration and frequency of fast gaps may in some cases be limited by the recording bandwidth, missed-event correction methods, or the occurrence of blocking events in high agonist experiments. Many of the problems that occur result in underestimates of the rates of the channel conformational changes.

The comparison of quantitative estimates of rate constants made in different studies can be a problem for two main reasons. First, there is the problem of correctly interpreting components of the

open and closed time histograms in terms of scheme 1. Second, although only incomplete data are available, it is clear that several rates depend on experimental variables such as membrane potential, temperature and the ion composition of the bath solution. These conditions vary among studies and the appropriate correction factors are not known. This section and the following one will emphasize data obtained under comparable conditions, and will seek to extract relatively general conclusions.

Results described below include observations from both embryonic and adult forms of the neuromuscular AChR (*see* III). A number of studies indicate that the activation behavior of these receptors differs (Auerbach & Lingle, 1987; Jaramillo & Schuetze, 1988), and it should be kept in mind that some of the differences between studies may reflect which form of the receptor is being studied.

C. Experimentally Measured Duration Distributions Show Multiple Components

1. Open Interval Durations Show Two Components

The distribution of the duration of open intervals has in general at least two components, although scheme 1 would predict only one. The shorter duration component has a mean time constant of about 100–400 μsec. Typically these brief openings are isolated events. Short duration openings have been observed for virtually every neuromuscular ACh receptor that has been examined. There is evidence that at least some, and in a few cases, most, of the short duration openings result from opening of singly liganded ACh receptors. Postulating that the channel can open when the receptor is monoliganded requires that scheme 1 be extended to

$$\begin{array}{ccccc} A + R & \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} & AR & \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} & A_2R \\ & & \alpha_1 \uparrow\downarrow \beta_1 & & \alpha_2 \uparrow\downarrow \beta_2 \\ & & AR^* & \underset{k'_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} & A_2R^* \end{array} \qquad (2)$$

That monoliganded receptors can open is inferred from the observation that the ratio of long duration openings to short duration openings increases linearly over the range of low agonist concentrations in some preparations (up to about 1 μM ACh (Colquhoun & Sakmann, 1985; Papke et al., 1988; Kidokoro & Rohrbaugh, 1990); up to 20 μM carbachol (Jackson, 1988)). Available results do not completely eliminate the possibility that short and long openings might arise from independent populations of receptors with different concentration-response characteristics. However, this seems unlikely since the ratio of short and long openings is fairly constant from one patch to another at a given agonist concentration (Colquhoun & Sakmann, 1985; Kidokoro & Rohrbaugh, 1990), and temporal correlations between brief and long openings are most consistent with the idea that a single AChR produces both (Sine & Steinbach, 1984*a*).

Several observations remain at variance with the view that short openings reflect exclusively the occurrence of singly liganded openings. For example, clusters of short openings can be observed at agonist concentrations above 20 μM (Auerbach & Lingle, 1986; *see* Fig. 1*A*). These observations would seem to reflect either a distinct mode of channel opening or a distinct channel variant (*see* Sections III and IV). Furthermore, in some preparations the frequency of brief openings at higher agonist concentrations greatly exceeds that which would be expected based solely on scheme 2 (Sine & Steinbach, 1984*a*; Colquhoun & Sakmann, 1985).

In addition, when a receptor is activated by a single tethered agonist molecule both short and long duration openings are observed (Chabala & Lester, 1986). Finally, spontaneous (agonist-independent) activation of the ACh receptor has been reported to result in both short and long duration openings (Jackson, 1986). Clearly, the issue of the factors that determine open channel lifetime extends beyond whether one or two agonist sites are occupied.

2. Burst Durations Show Two Components

The distributions of burst durations at lower agonist concentrations broadly mirror the properties of the open interval distributions. In most cases, two distinct components are observed. The faster component largely corresponds both in duration and in number with the short duration open times, indicating that the great majority of brief openings occur as isolated openings. The longer component of the burst length distribution appears to be composed primarily of a sequence of long duration openings.

As outlined above, according to scheme 1 at low agonist concentration three reaction rate constants are predicted to participate in defining the average burst duration. Thus, differences in burst duration may arise from differences in the agonist dissociation rate (k_{-2}), in rates of channel isomerization (α and β), or from all three.

Quite apart from its significance for the evaluation of reaction rates, the burst, as defined under conditions of low agonist concentration, has a

Table 1. Properties of closed intervals

	τ_1 (μsec)[a]	τ_2 (msec)	τ_3 (msec)	τ_4 (msec)
Low [ACh] (≤100 nM)				
Frog NMJ;[1] adult AChR	20 (96.6%)	0.51 (3.4%)	—	
Rat muscle;[2] embryonic AChR	89 (≈71%)	1.09 (≈23.8%)	14.4 (5.2%)	
BC3H1; mouse[3] embryonic AChR	48 (86.4%)	0.9 (13.6%)	—	
Xenopus;[4] embryonic AChR	20–40 (≈80%)	0.2–1.0 (20%)	—	
High [ACh]				
BC3H1; mouse[5] embryonic AChR	40 (66.3%)	0.24 (8.4%)	1.34 (8.4%)	11.96 (12%)
Xenopus;[6] embryonic AChR	25 (65%)	0.25 (2%)	3.26 (33%)	—

Notes:
[1] Colquhoun & Sakmann, 1985. 12°C; −120 mV; bandwidth 4–9 kHz; [ACh] 100 nM.
[2] Jaramillo & Schuetze, 1988. 13°C; −120 to −150 mV; bandwidth 4–7 kHz; [ACh] 10–100 nM.
[3] Sine & Steinbach, 1986*b*. 11°C; −100 mV; bandwidth 7.2 kHz; [ACh] 100 nM.
[4] Kidokoro & Rohrbaugh, 1990. 11–16°C; −175 mV; bandwidth 5 kHz; [ACh] 100–200 nM.
[5] Sine & Steinbach, 1987. 11°C; −120 mV; bandwidth 7.2 kHz; [ACh] 20 μM.
[6] Auerbach & Lingle, 1987. 23°C; −120 mV; bandwidth 5 kHz; [ACh] 10 μM.
[a] Each entry gives the time constant and relative prevalence of an exponential component in the distribution of closed times. At low [ACh] only closed times *within bursts* are listed, at high [ACh] only closed times *within clusters*. A dash indicates a component was not seen.

broader significance in terms of the physiology of synaptic transmission. Following evoked release from the nerve ending, free ACh is rapidly removed by binding and hydrolysis (Magleby & Stevens, 1972). Thus the mean duration of the long burst component closely approximates the average decay rate of the synaptic current at both developing and adult neuromuscular junction (Kullberg & Owens, 1986).

3. Closed Interval Distributions also Show Multiple Components

At low agonist concentrations, at least two kinds of gaps are usually discernable within bursts (*see* Table 1). One gap typically has a mean duration of less than 100 μsec ("brief" gaps). A second gap of about 1 msec duration ("intermediate" gaps) contributes about 3–20% of closures within bursts at low agonist concentrations. Long duration closures, separating bursts, are thought to include time intervals delineating the activity of an individual receptor from another; these gaps will not be discussed further. Multiple kinds of closed gaps within bursts have been seen in receptors from many sources—chick muscle (Auerbach & Sachs, 1983, 1984), clonal BC3H1 receptors (Sine & Steinbach, 1986*b*; Papke et al., 1988), rat muscle (Jaramillo & Schuetze, 1988) and amphibian ACh receptors (Colquhoun & Sakmann, 1985; Auerbach & Lingle, 1987; Igusa & Kidokoro, 1987; Rohrbaugh & Kidokoro, 1990). This is true both for embryonic and adult types of receptors (Auerbach & Lingle, 1987; Jaramillo & Schuetze, 1988; Kidokoro & Rohrbaugh, 1990).

It has not been possible to determine unambiguously the mechanistic origin of a particular gap. One approach has been to determine whether a particular kind of gap shows a dependence on the nature of the agonist molecule used to activate the channel (Colquhoun & Sakmann, 1985; Sine & Steinbach, 1986*a*,*b*; Papke et al., 1988). The argument is that, if an observed gap is the A_2R gap, it might be expected to exhibit pronounced dependence on the nature of the agonist. A weaker agonist may have a faster k_{-2} and a slower β. A second approach to attaching significance to a particular gap is to choose that gap which yields estimates of β/α that are most consistent with the expectations derived from the saturating value of p_{open} observed at higher agonist concentrations (Colquhoun & Sakmann, 1985; Auerbach & Lingle, 1987). Finally, some gaps have been shown to be transitions to a subconduc-

tance state (Auerbach & Sachs, 1983, 1984; Jaramillo & Schuetze, 1988) and are therefore unlikely to represent the A_2R gap.

Only two studies have attempted to examine the behavior of gaps within clusters of channel openings activated at high agonist concentrations (Auerbach & Lingle, 1987; Sine & Steinbach, 1987). The purpose of such experiments is twofold: first, to identify gaps which are clearly associated with receptor activation by virtue of their concentration dependence, and second, to define a limiting value for the concentration-dependent gap at saturating agonist concentrations. On BC3H1 cells at 11°C, four distinct gaps within clusters were identified (*see* Table 1). Overlap of different components across the concentration range examined made it difficult to identify gaps associated with receptor activation (Sine & Steinbach, 1987). However, a gap that behaved in accordance with previous low concentration results (Sine & Steinbach, 1986*b*) was identified. For *Xenopus* myocytes at room temperature, three distinct closed gaps appear within clusters of openings activated at concentrations of ACh ranging from 10 to 200 μM. The duration of one closed component exhibited a clear decrease in duration with an increase in ACh concentration. The limiting value of this gap at high [ACh] should provide an estimate of β, independent of the estimates obtained at low agonist concentration. However, as described above, estimates of the limiting value may be distorted by the presence of gaps due to channel block. As a result, current estimates of β' from this method are probably unreliable.

D. Our Ability to Interpret Duration Distributions is Limited

1. Interpretation of the Duration Histograms Requires a Kinetic Model

Interpretation of the open and closed time distributions requires some model framework. Most analyses have been performed using equations derived from scheme 1, with small modifications to account for brief openings or excessive numbers of closed time components. So the following sections summarize and compare estimates for the rate constants shown in scheme 1. Emphasis is placed on the interpretation of closed times, and the resulting estimates of rates for channel opening and agonist association and dissociation.

Overall, the use of scheme 1 for the analysis of the available data appears to be justified. In particular, the variation with agonist concentration of the amplitude of whole-cell responses, the frequency of occurrence of long duration bursts, and the duration of the closed time components all indicate that the greater part of the endplate current passes through receptors that have two ACh molecules bound. Furthermore, interpretation of the data in terms of scheme 1 has resulted in consistent descriptions of data obtained over a reasonable range of concentrations, and the scheme can be used to describe activation elicited by either steady state or transient applications of ACh. That there are features of the data not accounted for by scheme 1 means that the kinetic scheme cannot be considered to be a full description of activation. The scheme is used because it is the simplest available model that describes the major features of AChR activation.

Since scheme 1 does not provide a complete description of most data sets, it must be kept in mind that parameter estimates derived in terms of this model are tentative.

2. Gaps Must Have Meaning

The critical question is how particular gaps are related to reaction steps in hypothetical receptor reaction schemes. In most cases, the fastest observable gap has been interpreted to represent the A_2R gap, while the intermediate component has been interpreted either as the expected intermediate duration activation gap (an "AR gap") (Colquhoun & Sakmann, 1985) or some other closed state that is related directly to the open state but does not participate in the activation pathway (Auerbach & Lingle, 1987; Jaramillo & Schuetze, 1988). A notable exception to this interpretation is the work of Sine and Steinbach (1986*b*) who interpreted their intermediate gap component as an A_2R gap, and the fast gap as a nongating gap. It is worth considering some of the reasons various authors developed in making their selections, since it helps to illuminate the limits of our current understanding.

Those authors selecting the brief gap as the important A_2R gap have used several criteria. First, in many cases the estimates of $\beta/(\alpha + \beta)$ predicted from the characteristics of the fast gap correlate better with high agonist concentration estimates of p_{open} (Colquhoun & Sakmann, 1985; Auerbach & Lingle, 1987; Papke et al., 1988; Kidokoro & Rohrbaugh, 1990). However, in BC3H1 cells, even the intermediate gap predicts a maximal p_{open} in excess of 0.9. Second, the rise time of miniature endplate currents is more consistent with the high estimate of β obtained from the assumption that fast gaps are the A_2R gap. Finally, Colquhoun and Sakmann (1985) showed that with a series of agonists including two more potent than ACh and one weaker than

ACh, the kinetic estimates for β, α and k_{-2} led to predictions of agonist potency consistent with independent estimates.

Work on BC3H1 cells is also of interest in this regard. Sine and Steinbach (1986*a*) took advantage of a presumed weak agonist (dimethyl-D-tubocurarine; DMT) which would be expected to be of high affinity but low efficacy. It binds to the receptor quite tightly but results in no measurable flux of $^{22}Na^+$ (Sine & Taylor, 1981). Thus, β would be expected to be quite low and β/α should be relatively small. With the higher resolution of single channel records, DMT can be shown to activate AChRs at a low frequency compared to ACh. Surprisingly, with DMT and ACh the frequencies and durations of the fast gap component in BC3H1 cells are quite similar. As a consequence, it is quite reasonable to argue that the fast gap observed in BC3H1 cells probably does not reflect the A_2R gap. From this basis, their reasoning suggested that intermediate gaps were more likely to represent A_2R gaps. However, recent evidence using fast agonist applications to patches of BC3H1 membrane suggests that the faster gap would result in more likely estimates of receptor activation rates (Liu & Dilger, 1991).

ACh receptors expressed in fibroblasts using cDNA sequences for subunits derived from *Torpedo* electric organ (*see* section IV) are a noteworthy exception to this confusing situation. The analysis of closed time distributions by Sine and colleagues (Sine et al., 1990) showed a single component within bursts at low concentrations. Furthermore, across a wide concentration range all of the components could be described by scheme 1 with a single set of estimates for rate constants.

For neuromuscular AChR, the prevailing view is now that intermediate gaps at low agonist concentrations, at least with strong cholinergic agonists, probably do not represent the A_2R gap but are channel closures to an undefined closed state. Furthermore, the properties of the fast gap have been fairly consistent in predicting channel behavior at saturating agonist concentrations. However, in the absence of an independent test, the question still remains, whether the fast gap truly represents the gating gap. It is still possible that the true gating gap has not yet been evaluated. In particular, what are the fast gaps when the agonist is a molecule like DMT?

In this regard it is worth noting that Colquhoun and Sakmann (1985) observe that in a substantial number of experiments two exponential components were required to describe adequately the gap distributions over the range of 30–300 μsec. This raises the question whether brief gaps really are a single population of events.

3. The Channel Opening Rate is of Critical Importance

A central problem in obtaining accurate quantitative estimates of rates is that uncertainties in one estimate propagate into estimates of others. As an example, for low agonist concentrations, uncertainties in properties or interpretation of brief closed periods result in uncertainties in estimates of α, β, and k_{-2}. As a consequence, one particular rate constant, the channel opening rate, β, has been of key importance in attempts to understand AChR activation. β is, in principle, the rate constant most susceptible to independent measurements. It can be estimated from single channel data at both low and high agonist concentrations. At high agonist concentrations, in theory, it can be estimated independently of either α or k_{-2}. Furthermore, β can be estimated from fast agonist application methods (Liu & Dilger, 1991). Once β is well defined, both k_{-2} and α can then be obtained.

Evaluated from the kinetic analysis of single channel data, the various estimates of β show a perhaps surprising variability (*see* Table 2). However, as outlined above, a number of factors can compromise such attempts. Assuming the fast gap is the A_2R gap, the estimate for β from embryonic mammalian AChRs at 11°C is in excess of 6,000 sec^{-1} (Sine & Steinbach, 1986*b*; Jaramillo & Schuetze, 1988). For the primary mode of embryonic *Xenopus* channels, the best available estimate suggests that β probably exceeds 25,000 sec^{-1} (Kidokoro & Rohrbaugh, 1990). For adult amphibian receptors at synaptic membranes, β has been estimated at about 30,000 sec^{-1} (Colquhoun & Sakmann, 1985). One estimate for β for the large conductance form of mammalian receptor is 23,000 sec^{-1} (Jaramillo & Schuetze, 1988). These data suggest that the opening rate for adult receptors appears somewhat faster than β for embryonic receptors. Estimates of β in excess of 20,000 sec^{-1} are consistent with estimates obtained from analysis of the rising phase of synaptic currents (Land et al., 1981).

Recent advances in fast agonist application procedures mean that an independent method of verifying channel activation is now available. This method avoids many of the uncertainties and assumptions inherent in measurement and evaluation of interval durations in single channel data. Results based on fast application of ACh to outside-out patches from BC3H1 cells at 11°C place a lower limit on β of about 14,000 sec^{-1} (Liu & Dilger, 1991). Fast application of ACh to patches containing mouse adult or fetal receptors also indicate that the opening rate for adult reception is higher than

Table 2. Estimates of activation parameters

	Cluster p_{open}[a]	β/α and p_{open}[b] from rates	β (sec^{-1})[c]	k_{-2} (sec^{-1})[d]	α (sec^{-1})[e]
Frog NMJ[1] Adult AChR	0.98	43 (0.977)	30,600	8,150	714
Xenopus[2] embryonic AChR	>0.94 (100 μM)	77.4 (0.987)	26,400	16,900	341
Rat muscle[3] embryonic AChR		68.5 (0.986)	5,755	6,356	84
BC3H1[4] mouse embryonic AChR	0.93	13.7 (0.93)[f] from intermediate gaps;	480, 321;	1,000, 860;	35
		224.2 (0.996) from fast gaps	13,904	6,500	62

Notes:
[1] Colquhoun & Sakmann, 1985: Ogden & Colquhoun, 1983; Colquhoun & Ogden, 1988. 11°C; −120 mV; bandwidth 4–9 kHz.
[2] Kidokoro & Rohrbaugh, 1990; Igusa & Kidokoro, 1987. st. 41–42 embryonic major mode; 11–16°C; −175 mV; bandwidth 5 kHz.
[3] Jaramillo & Schuetze, 1988. 11°C; −120 mV; bandwidth 4–7 kHz.
[4] Sine & Steinbach, 1986*b*, 1987. 11°C; −100 mV; bandwidth 7.2 kHz.
[a] Fraction of time spent open in a cluster at a saturating [ACh].
[b] Ratio of opening to closing rate (estimated at low concentration), and the calculated p_{open} from the rates ($\beta/(\beta + \alpha)$).
[c] Estimated channel opening rate: *see* scheme 1.
[d] Estimated dissociation rate for the first ACh molecule to unbind from a doubly liganded receptor.
[e] Estimated channel closing rate.
[f] Values calculated from intermediate (upper line) and brief (lower line) component of closed times within bursts at low [ACh].

for fetal (at 22°C, about 50,000 sec^{-1} compared to 15,000 sec^{-1}; Maconochie & Steinbach, 1992).

4. What Is the Affinity of the Receptor for Agonist?

The answer to this question is dependent upon whether the two binding steps are equivalent or distinguishable. As yet, this issue cannot be considered to have been resolved. Biochemical and physiological data indicate that each ACh receptor possesses two binding sites for ACh and that most receptors with open channels have ACh bound to both sites. The question then arises whether or not the two sites are equivalent (e.g., Karlin, 1967; Dionne et al., 1978; Colquhoun & Sakmann, 1985; Sine et al., 1990). There are data, outlined below, which suggest that ACh may bind to the two sites on the AChR with different affinities. Affinity differences could arise in two ways. The simplest is if the two sites differ even before any ligand is bound ("distinguishable sites"). An alternative mechanism is that the sites are initially identical, but binding of agonist allosterically alters the affinity of the second binding site ("cooperative sites"). AChR with positively cooperative sites would have a steeper binding curve than predicted for AChR with identical and noninteracting sites. Receptors with either initially distinguishable sites or negatively cooperative sites show flatter binding curves and cannot be differentiated from each other on the basis of equilibrium binding data alone. However, AChR with initially distinguishable sites and negatively cooperative sites can be differentiated by some biochemical manipulations (Sine & Taylor, 1981).

Biochemical data obtained from both the BC3H1 mouse muscle receptor (Sine & Taylor, 1981) and the *Torpedo* receptor (Neubig & Cohen, 1980) have clearly demonstrated, from the binding of some curariform antagonists, that the two sites for ACh are distinguishable. However, it has been shown that agonist binding sites in both the human skeletal muscle AChR expressed by the TE671 cell line (Sine, 1988) and in AChR in *Electrophorus electroplax* (Prinz & Maelicke, 1983) exhibit much less difference in affinity for these antagonists. Overall, the data support the view that the binding of curariform antagonists is at distinguishable sites, but there is only limited biochemical evidence that the

affinity of the two binding sites differs for agonists (Blount & Merlie, 1989).

If the nicotinic receptor really has the pronounced nonequivalence seen in binding of antagonists, then why is this not apparent in biophysical studies? Many studies (e.g., Dionne et al., 1978; Colquhoun & Sakmann, 1985; Colquhoun & Ogden, 1988; Auerbach & Lingle, 1987; Sine & Steinbach, 1987) have found that kinetic data were well described by assuming that the two agonist-binding sites either had equivalent affinities or showed slight positive cooperativity in binding. Two more recent studies, however, have both reported evidence for initially distinguishable sites. Jackson (1988) studied fetal-type receptors on mouse myotubes and analyzed the concentration dependence of the frequency of brief and long openings. The frequency of brief openings initially increased linearly with carbachol concentration but saturated at about 20 μM. The frequency of long openings, on the other hand, increased supralinearly with carbachol concentration and showed little sign of saturation at 20 μM. The interpretation is that the first binding site is saturated at a lower carbachol concentration than the second (perhaps as much as 100-fold lower), implying distinguishable sites. However, the shape of the frequency versus concentration curve is not very well defined near 20 μM. Sine et al. (1990) analyzed the gap duration histograms for *Torpedo* receptors expressed in fibroblasts across a wide concentration range and reached a similar conclusion. It is not known how representative of muscle ACh receptors the *Torpedo* electroplax receptors actually are. However, biophysical data now seem to indicate that neither positive cooperativity nor equivalence of receptor binding sites is the correct model, but that the AChR either has initially distinguishable sites or shows negative cooperativity in binding ACh.

A possible explanation for the uncertainty in this area is that measurements of whole cell currents and ion fluxes did not allow for the effects of either desensitization or channel block. For example, a receptor showing strong negative cooperativity or having distinguishable sites would be expected to have dose-response relationship with a Hill slope of around 1 at high agonist concentrations. However, Sine et al. (1990) have illustrated how channel block coupled with an activation process involving large affinity differences can result in dose-response curves with apparent Hill coefficients approaching 2.

The relative affinity of the two binding sites does not affect the way k_{-2} is determined. A value for k_{-2} in excess of about 6,000 sec^{-1} at 10°C (Table 2) is observed for all preparations, being perhaps somewhat faster in amphibians. Few estimates of k_{+2} have been made from experimental data, and it is often assumed that the association rate is limited by the diffusion of agonist to the receptor (a rate constant of about 10^8 M^{-1} sec^{-1}). With this value for k_{+2}, the second agonist binding step has a microscopic affinity of about 60–150 μM for ACh.

The concentration at which channels are open about 50% of the time in the absence of desensitization is typically in the range of 10–25 μM. It is worth noting that a half-maximal response can be elicited at a concentration below the microscopic affinity of either binding step. The dissociation constant for the second agonist binding step of scheme 1 is defined as $K_2 = k_{-2}/k_{+2}$, or the concentration of agonist at which $[A_2R] = [AR]$. However, because of the large ratio of β/α most doubly liganded receptors will have open channels and the distribution of receptors among the various states will be shifted to the right in scheme 1. Hence, half of all receptors can have open channels (and be doubly liganded) at a concentration of agonist that is lower than the dissociation constant for the second ligand-binding step.

Moreover, the apparent dissociation constants obtained in equilibrium binding assays for agonists to AChR also will show a higher affinity than that for the binding steps shown in scheme 1. In this case the disparity results from the fact that equilibrium binding data are strongly influenced by agonist-induced desensitization of the receptor, which results in an increase in the affinity of the receptor for agonist.

E. Where We Are and What Needs to be Done

From the perspective of the synaptic physiologist, the broad features of activation of ACh receptors can be considered to be well understood. There appears to be general agreement in the following areas.

At saturating concentrations of ACh, in the absence of desensitization and channel block, strong agonists can open over 95% of the available channels, implying that β is substantially greater than α. Once a channel is doubly liganded, it will undergo on average several cycles of opening and closing before the agonist dissociates, implying that β is similar to or greater than k_{-2}. The concentration of ACh that results in activation of 50% of the available channels is somewhat lower for fetal-type channels than for adult-type channels, being about 5–10 μM and 15–25 μM, respectively. There is also general agreement that β is fast (e.g., at 11°C,

10,000 sec^{-1} for mammalian embryonic receptor and 30,000 sec^{-1} for amphibian adult receptor). However, these estimates are dependent on the measurement of gaps in single channel records which may be limited by systematic factors.

Despite general agreement in the areas listed above, a number of critically important questions require further investigation.

1) What is the structural basis for brief and long duration openings? If monoliganded receptors produce brief openings, why is this open state less stable than the doubly liganded and open state? Alternatively, for those brief openings which apparently arise from the doubly liganded AChR, what is the basis for this different open state?

2) How rapidly do channels open? Furthermore, what physical mechanism is likely to underlie protein conformational changes with rates of the order of 30,000 sec^{-1}?

3) What is the affinity of nondesensitized receptors for agonist, and do the two agonist-binding sites differ in their affinity for agonist?

4) Can the classical model really account for the data? In some respects the classical model is analogous to a billiard ball model of elementary particles, in that it ignores the possibility of intervening states of the ACh receptor (for example a conformational change occurring after each binding step but preceding actual channel opening). Recent studies (A. Auerbach, 1992) suggest that such a model can produce statistically better descriptions of the data than a version of scheme 1 with the same number of parameters. Scheme 1 (and the modification just mentioned) contain a small number of distinguishable kinetic states. Overall, models based on Markov processes such as this have provided better descriptions of the data than fractal or diffusion gating processes (Korn & Horn, 1988; McManus et al., 1988, 1989; Horn & Korn, 1989; Liebovitch, 1989).

Further progress will likely result from two advances. For single channel records, it will be necessary to apply improved analytical methods in which multiple data sets will be fit directly with particular kinetic models. This will provide better parameter estimates for each model and will allow valid statistical comparisons among models. Furthermore, independent estimates of rates must be obtained; at present, the most promising approach is the fast-flow concentration jump technique to provide estimates for the channel opening rate.

III. There Are Several Kinds of Neuromuscular AChR

A common problem in the study of membrane ion currents is to determine how many distinct kinds of channels are present in the membrane. Even after a single class is identified pharmacologically, for example the nicotinic AChR, the data may show an unfortunate degree of complexity. For example, the kinetic behavior often cannot be accounted for by the assumption that there is only one kind of channel operating in accordance with a simple kinetic scheme (e.g., scheme 1).

A. Embryonic and Adult ACh Receptors Are Different

One outcome of the application of molecular biological methods to the study of ionic channels has been the realization that most ion channels are members of large families of related ion channels. For example, it is now clear that there are a large number of different forms of subunits that participate in formation of neuronal nicotinic AChRs (Steinbach & Ifune, 1989). In the case of vertebrate muscle, there appears to be a more limited set of subunits involved in the formation of AChRs.

Early work on denervated and developing muscle suggested that two kinetically distinct forms of the AChR might be present in muscle. In particular, at immature junctions and in extrajunctional regions of denervated adult muscle fibers, acetylcholine receptors were expressed which had a longer open channel lifetime than receptors at normal adult junction. Subsequently, in both mammalian and amphibian preparations, two primary conductance classes have been observed (Hamill & Sakmann, 1981; Leonard et al., 1984). The smaller class (conductance of 25–40 pS, called γ_{40}) of channel, which is predominantly associated with embryonic or denervated muscle, typically produces bursts of 2–10 msec mean duration while the large class (conductance of 50–70 pS, called γ_{60}) is associated with shorter duration bursts (0.5–1.5 msec). A compelling study (Mishina et al., 1986) has provided a plausible structural explanation for the switch in primary conductance levels between embryonic and adult muscle: during development, expression of a γ subunit is reduced while expression of an ε subunit is increased. Because of the difference in conductance, the two kinds of receptor can be easily distinguished in single channel recordings.

B. There is Complexity in Kinetic Behavior within a Single Conductance Class

A second kind of complexity (often called heterogeneity) is the occurrence of kinetically distinct patterns of behavior within a single conductance class (Auerbach & Lingle, 1986; *see* Fig. 1). It is clear

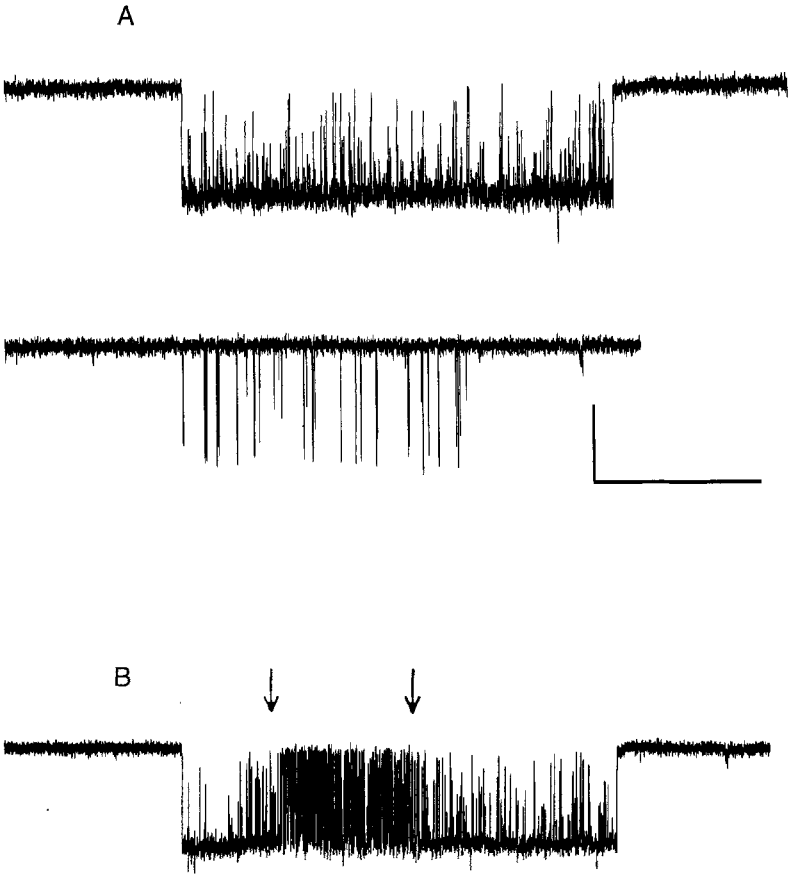

Fig. 1. Aspects of ACh channel kinetics not accounted for by standard activation schemes. (*A*) Examples of two clusters of openings activated by 100 μM ACh recorded from a cell-attached patch on a BC3H1 clonal muscle cell are shown. The cluster in the top trace is representative of the more usual sort of behavior of AChRs. The lower trace indicates that short openings can also be grouped in clusters. The mean open time for 366 detected openings in the first cluster was 3.22 msec and the mean closed time was 0.14 msec (p_{open} = 0.96). The mean open time for 36 openings in the bottom cluster was 0.74 msec and the mean closed time was 22.30 msec (p_{open} = 0.03). (*B*) An example of putative mode switching within a cluster of openings activated by 50 μM ACh on a BC3H1 clonal muscle cell is shown. The cluster begins with a relatively long open time activation mode, after about 290 msec switches to a mode of shorter open times and longer closed times, and then after about another 420 msec returns to a longer open time pattern of behavior. The arrows indicate approximate times at which a change in activation behavior occurred. For the first 290 msec of the cluster, the mean open time for 50 events was 5.77 msec and mean closed time was 0.25 msec with a p_{open} of 0.96. From 290 through 710 msec, the mean open time for 371 events was 0.61 msec and mean closed time was 0.49 msec with a p_{open} of 0.55. From 710 through 1300 msec, the mean open time for 145 events was 3.83 msec and mean closed time was 0.25 msec with a p_{open} of 0.94. The p_{open} for the entire cluster was 0.82. This cluster followed the end of the preceding cluster by 3 sec and the next subsequent cluster occurred after another 49 sec. Vertical calibration: 2.5 pA; horizontal calibration: 480 msec. Records in *A* and *B* were digitized at 50 kHz, analyzed with 5 kHz filters, and plotted with 1 kHz filtering.

that this type of complexity is a feature of ACh receptors in most muscle preparations that have been examined, and many authors point out that at least some patches had channels with unusual kinetic behavior (Jaramillo & Schuetze, 1988; Jackson, 1988; Colquhoun & Sakmann, 1985; Sine & Steinbach, 1984*a*). The significance of and mechanistic basis for this behavior remain largely a matter of speculation. Since kinetic heterogeneity can easily be overlooked, the extent of heterogeneity is also not clear. Particularly in experiments at low agonist concentrations, in which the mean apparent open time and the single channel conductance are the primary fingerprints used for identification of the channel type, it is difficult to assess whether channels of different agonist affinities or opening rates may be present. At higher agonist concentrations, in which several cycles of opening and closing of a single ion channel protein can be clearly resolved, kinetic heterogeneity can be observed more readily.

The clearest example of kinetic heterogeneity was observed in cultured *Xenopus* myocytes (Auerbach & Lingle, 1986; Leonard et al., 1988*b*). In this system, single channel openings at high ACh concentrations appear to occur in "clusters" of openings, with long closed periods separating clusters. Each cluster of openings was assumed to indicate the activity of a single ion channel only (Auerbach & Lingle, 1986). At least three distinct "modes" of behavior could be distinguished for both γ_{40} and γ_{60} conductance classes. Using p_{open} to distinguish between different kinetic modes, it was observed that both the mean open time and the mean closed time differed between the different modes. At low agonist concentrations, distributions of open intervals could often be described by three components, which correlated well with the modes observed at high agonist concentrations. Assuming the standard activation model for the ACh receptor (scheme 1), these results imply that either separate ACh receptors or a single receptor functioning in different modes differ not only in their channel closing rates but also in the transitions affecting their apparent opening rates.

There are also indications of kinetic heteroge-

neity in the BC3H1 clonal muscle cell line (Sine & Steinbach, 1984*a*, 1987; Fig. 1). The mean open time for clusters activated at high agonist concentration differ from each other by a much greater degree than can be explained by sampling from a homogeneous population, suggesting that individual AChRs may differ (Sine & Steinbach, 1987).

Recently, Gibb et al. (1990) have shown that expression of mRNA from BC3H1 cells in *Xenopus* oocytes can result in the appearance of kinetically distinct types of ACh-activated channels. Data from a limited number of oocytes suggest that receptors expressed by a given oocyte appeared to have similar kinetic properties while other oocytes had channels which were different. This result suggests that functional heterogeneity can occur even with a presumably defined set of receptor subunits. Gibb et al. tentatively suggested that post-translational modifications might be the cause of the differences.

It is not clear to what extent heterogeneity in channel kinetic properties occurs at fully differentiated neuromuscular junctions. In their analysis of single channel properties activated by lower agonist concentrations at the mature frog neuromuscular junction (NMJ), Colquhoun and Sakmann (1985) point out that there are a number of unexpectedly long bursts not accounted for by the bulk of their observations, and Mulrine and Ogden (1988) state that at adult rat NMJ, both high and low p_{open} clusters occur at high [ACh]. Recent work of Dionne (1989) is also consistent with the possibility that kinetically distinct forms of a single conductance class of channel may be present in adult snake endplate membrane.

C. ACh Receptors Show Rare Switches in Kinetic Mode

The critical question that arises concerning the occurrence of kinetic heterogeneity is whether it is the result of the complex behavior of a single receptor protein or indicative of multiple forms of AChRs. This question has been addressed for a number of other ion channels. Complexity in kinetic behavior has been observed for voltage-dependent sodium channels (Patlak et al., 1986), voltage-dependent calcium channels (Hess et al., 1984; Nowycky et al., 1985; Bean, 1989), insect glutamate receptors (Patlak et al., 1979), and voltage-dependent calcium-activated potassium channels (McManus & Magleby, 1988). For most of these cases, complex behavior has been observed in patches thought to contain only a single ion channel protein. As a result, the evidence is good that individual ion channel proteins can undergo relatively stable changes in the mode or pattern of kinetic behavior. In such cases, any activation model that is sufficient only to describe the behavior of the channel in one mode is insufficient to account for the behavior of the channel in another mode. For clusters of openings activated by ACh, it is essentially impossible to assert definitively that individual clusters with distinct kinetic behavior originate from the same receptor protein. Only in cases where changes in kinetic behavior may be observed within well-isolated clusters is it possible to suggest that different kinetic modes arise from a single receptor.

Muscle AChR showing occasional cases of apparent switching between kinetic modes within a single cluster of openings can be observed (Auerbach & Lingle, 1986; C.J. Lingle, K. Nakayama and J.H. Steinbach, *unpublished*, *see* Fig. 1*B*). The rare incidence of such events has precluded systematic investigation. Rapid applications of high ACh concentrations to excised patches containing single ACh receptors have shown instances of mode switching (A. Naranjo and P. Brehm, *personal communication*), raising the possibility for more complete analysis of the processes involved.

D. The Origin of Heterogeneity in Kinetic Behavior is Unknown

Several suggestions have been made to account for the observed kinetic heterogeneity of the AChR (Auerbach & Lingle, 1986; Gibb et al., 1990).

1) There may be as yet unidentified receptor subunits. In *Xenopus*, there is a recent report of an α_{1a} subunit (Hartmann & Claudio, 1990).

2) Functionally distinct channels may result from an abnormal stoichiometry or ordering of subunits. Expression studies (*see* IV, below) show that functional channels with differing properties may occur in the absence of a full complement of subunits.

3) Alternatively, functionally distinct channels may reflect differences in covalent or post-translational modification. The acetylcholine receptor has several potential phosphorylation sites. At present, such sites have been implicated in desensitization processes (Huganir & Greengard, 1990), although this remains an issue of some debate (Wagoner & Pallotta, 1988; Reuhl et al., 1989). But, it has not yet been shown that any covalent modification can reversibly change the gating behavior of the acetylcholine receptor, although inhibition of the normal glycosylation of the ACh receptor channel has been shown to result in channels of altered function (Covarrubias et al., 1989).

4) Finally, relatively stable changes in the state of individual ion channel proteins, perhaps analogous to transitions into and out of desensitized states might underlie heterogeneity. A change in kinetic behavior of ACh receptors following patch excision has been described (Covarrubias & Steinbach, 1990).

Unfortunately, at present there are no procedures which have allowed experimental manipulation of the frequency of occurrence or nature of the observed heterogeneity.

The physiological importance of functional heterogeneity remains unclear. However, the fact that the extent of heterogeneity is not known calls into question any kinetic data obtained under the assumption that channels behave as a kinetically homogeneous population. Since all analyses of stationary records (particularly, those at low concentrations) make this assumption, this overall issue must be resolved before valid estimates of kinetic parameters can be made.

IV. AChR Structure Alters AChR Activation in Poorly Understood Ways

The determination of the primary sequence of the muscle nicotinic AChR held out the promise that regions associated with channel function would be identified rapidly. For example, there is now a convincing body of evidence that the M2 membrane-spanning region and adjacent linking regions form the major part of the channel lining (Imoto et al., 1986, 1988; Charnet et al., 1990; Leonard et al., 1988*a*; *reviewed in* Dani, 1989). However, the available evidence indicates that gating processes are not similarly localized.

A. The AChR Is Composed of Structurally Related Subunits

The cartoons in Fig. 2 show relevant features of the structure of muscle AChR (for reviews, *see* Claudio, 1990; Stroud et al., 1990). The native muscle AChR is a large protein with an apparent molecular weight of about 350 kD, composed of five subunits (Fig. 2*A*). There are two copies of α and one copy each of β, δ and either γ (fetal) or ε (adult). The subunits are translated from separate mRNA transcripts, assembled in the endoplasmic reticulum, and processed in the endoplasmic reticulum and Golgi apparatus before being transported to the cell surface (Merlie & Smith, 1986). Each subunit seems to traverse the cell membrane at least four times (Fig. 2*B*), and the primary sequences of the subunits show a great deal of similarity, especially in the membrane-spanning regions (Fig. 2*C*). Models of the quaternary structure have the subunits placed around a central ion channel, like segments of an orange (Fig. 2*A*; *see* Karlin et al., 1983; Blount & Merlie, 1989), with their M2 regions lying closest to the channel. The N-terminal external domains contain covalently linked carbohydrate moieties, while the long cytoplasmic domains between M3 and M4 contain sites for protein phosphorylation. The N-terminal external domain of the α subunit contains the binding sites for ACh and the snake toxin, α-bungarotoxin (αBGT).

B. What Data on Function Are Needed in Structure-Function Studies?

The overall goal of studies of receptors expressed in nonmuscle cells has been to correlate observed changes in functional properties of AChR with changes in the AChR structure. Sections II and III have laid out some major considerations in terms of the kinds of data and analysis which lead to an understanding of the activation behavior of a population of receptors. The same sorts of experiments need to be performed with expressed channels, in order to understand the functional consequences of altered structure. A thorough analysis of burst kinetics at low agonist concentration should be performed to provide estimates of the rate constants for channel opening, closing and the dissociation of the first agonist molecule to unbind (α, β and k_{-2}). In addition, analysis of clusters should be done at medium to high agonist concentrations to provide estimates of the concentration required for half-maximal activation (EC_{50}), and to obtain the saturating p_{open} at high agonist concentrations. Studies at high agonist concentration also give essential information about the kinetic homogeneity of the population of receptors (section III). The limited data available suggest that kinetic heterogeneity is certainly no less of a problem with receptors expressed in nonmuscle cells, so some care must be used in deciding what the properties of the entire receptor population are.

It is disappointing that none of the studies with expressed AChR involve this set of experiments, although a few authors (e.g., Lo et al., 1990*a*,*b*) have performed a low concentration burst analysis.

It would also be valuable if different groups adopted standard conditions of temperature, membrane potential, saline composition, and recording bandwidth so that results from different studies could be compared directly.

In the absence of the critical kinetic data just discussed, the rest of section IV will focus on reported values of burst duration (sometimes given as

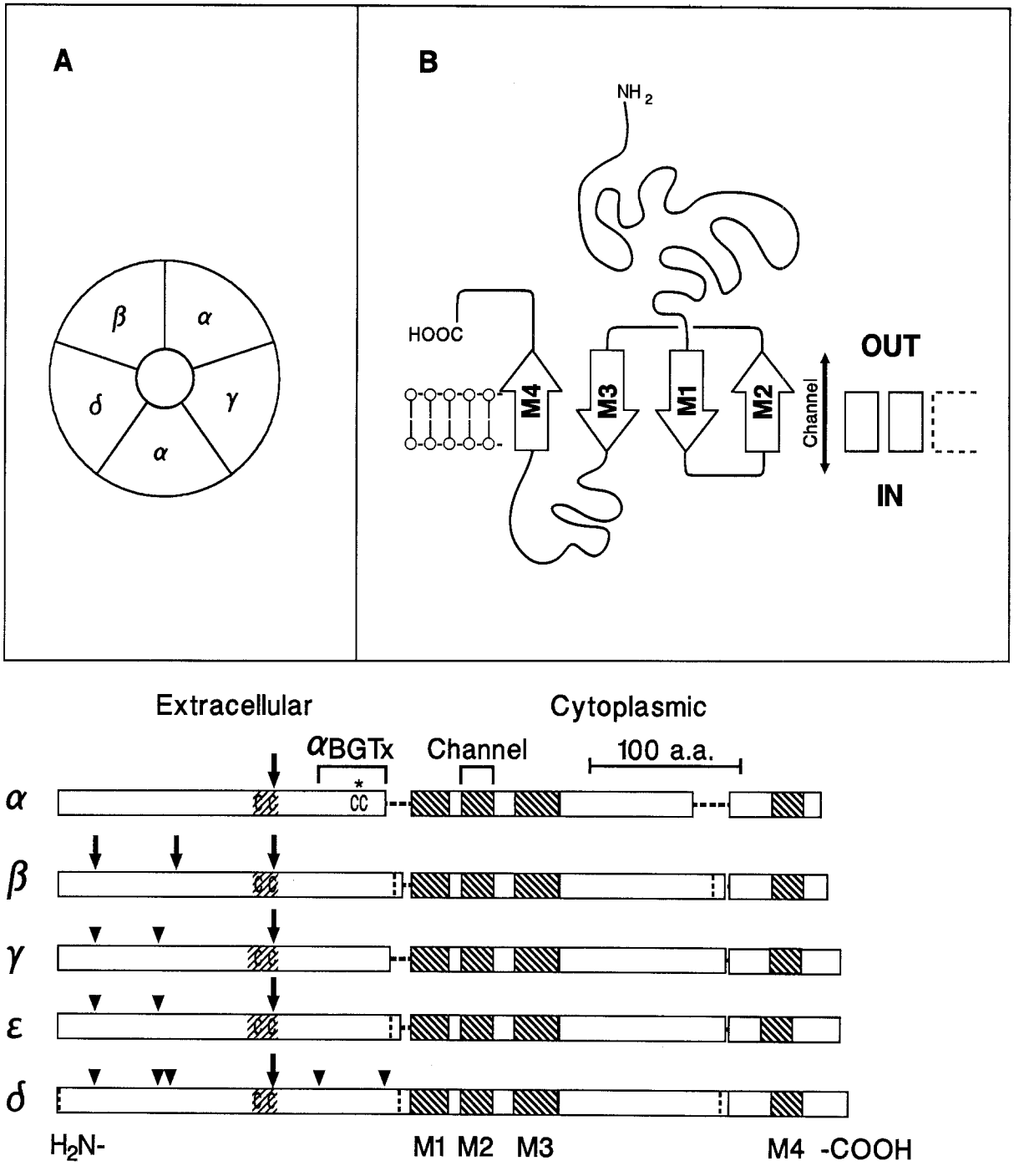

Fig. 2. Overview of muscle nicotinic AChR structure. (*A*) The muscle nicotinic ACh receptor is a pentamer with the subunits arranged around a central ion channel, which is formed by sequences contributed from each subunit (for references *see* text). (*B*) The N- and C-termini are both thought to lie on the external surface and there are thought to be four "membrane spanning helices" named M1–M4. The N-terminal external region comprises about one-third of each sequence, the C-terminal external region is quite small and the major cytoplasmic region is the loop between M3 and M4. (*C*) There are extensive similarities in primary amino acid sequence both between the subunits found in different species and the different subunits in a given species. The similarities are summarized in this cartoon. The membrane-spanning helices (heavy cross-hatch) are the regions of highest amino-acid similarity across subunits. A shorter region (light cross-hatch) is also more similar among subunits than the rest of sequence. Differences in lengths between subunits are emphasized by aligning similar regions vertically and using dashed lines to indicate "missing regions"; differences in length among subunits sequenced from different species are indicated by the vertical dashed lines. The N-terminal region includes glycosylation sites (arrows are sites found in all sequences of a subunit from different species, arrowheads are sites found only in some species), a conserved double cysteine found in the α subunit and thought to be the ACh-binding site, and a conserved region bounded by two cysteines in all subunits. The major cytoplasmic region contains sites for phosphorylation and differs most extensively among subunits.

"apparent open time"). As detailed in section II, the burst duration is determined by three rate constants (α, β, and k_{-2}), so differences in one or more of these parameters cannot be distinguished by the data available from most studies.

In the rest of this section, the recent evidence concerning the role of AChR structure in determining gating properties will be discussed. This will be divided into three portions: the expression of muscle nicotinic AChRs in nonmuscle cells, receptors with altered subunit composition and receptors with subunits containing experimentally introduced mutations.

A particular shorthand will be used in these sections: the phrase "*Torpedo* receptors expressed in oocytes" will be used instead of "receptors expressed following the introduction into *Xenopus* oocytes of cloned mRNA sequences coding for subunits of ACh receptors normally expressed by *Torpedo* electroplax." As will be mentioned below, there is a possibility that expression systems may produce receptors which include endogenous subunits or which lack one or more of the introduced subunits, so the shorthand is really a form of poetic license.

C. *Xenopus* Oocytes Express ACh Receptors Reasonably Faithfully

Most studies of receptors expressed in nonmuscle cells have involved the injection of synthetic cloned mRNA into *Xenopus* oocytes, but more recently cultured cells have been employed to express exogenous receptor subunits either on a transient or stable basis. The data in subsequent sections have been obtained following injections into *Xenopus* oocytes, but a brief mention of recent work with cultured cells will be made. A number of cultured nonmuscle cell lines have been used to express ACh receptors from *Torpedo* (Sine et al., 1990), mammalian fetal-type receptors (Claudio, 1990; Forsayeth et al., 1990; Gu et al., 1990; Phillips et al., 1991; Sine & Claudio, 1991) and adult-type receptors (Phillips et al., 1991). The properties of the expressed receptors appear grossly normal.

One interesting experiment with subunits stably expressed in fibroblasts addressed the question of what determines the binding affinity for small ligands, both agonists and antagonists (Blount & Merlie, 1989). By expressing the α (ACh-binding) subunit in combination with the γ or the δ subunit from mouse muscle, it was found that both the γ and the δ subunit associate with the α subunit in intracellular membranes, although none of the expressed subunits appear on the cell surface. Depending on whether $\alpha\gamma$ or $\alpha\delta$ pairs were formed, the ability of agonists and antagonists to block αBGT-binding to the α subunit showed either "high" or "low" affinity characteristics. The data provide an explanation for earlier observations that the two antogonist-binding sites on native receptors might not be equivalent and suggest that the γ and δ subunits either contribute a portion of the site or alter the structure of the subunit.

The *Xenopus* oocyte expression system has been used much more extensively than either stably or transiently transfected cultured cells. There are two reasons for concern about the fidelity of *Xenopus* expression systems. First, it has been shown that the glycosylation of *Torpedo* subunits expressed in oocytes is not identical to the native receptor (Buller & White, 1990*b*). Second, some pharmacological differences have been reported between voltage-gated Na^+ (Sutton et al., 1988) and K^+ (Zagotta et al., 1989) selective channels expressed in oocytes and *in situ*. The basis for the pharmacological differences is not known, but could reflect differences in post-translational processing, protein folding, or the presence or absence of additional channel-associated proteins expressed by excitable cells.

When the full complement of subunits is injected, ACh receptors are generated in oocytes with properties that usually are similar to those of the receptor *in situ* [*see* Mishina et al. (1986) for bovine $\alpha_2\beta\gamma\delta$ and $\alpha_2\beta\delta\varepsilon$ receptors and Yu et al. (1991) for mouse $\alpha_2\beta\gamma\delta$ receptors]. Few of these studies have explicitly considered the question of the homogeneity of the receptors, although most reports suggest that all of the expressed receptors are functionally identical, when they function at all.

Some more recent results reveal complexities or problems. Gibb et al. (1990) have reported that mouse AChRs expressed in oocytes using mRNA isolated from a clonal cell line (BC3H1 cells) show two distinct kinetic modes of behavior (*see* section III). Two recent reports indicate that subunit assembly may be heterogeneous in oocytes. Kullberg et al. (1990) and Jackson et al. (1990) present data suggesting that some AChRs are expressed that appear to be missing a subunit, even when mRNA for all four subunits are injected (*see below*). Finally, oocytes may express endogenous mRNA coding for ACh receptor subunits. Detectable levels of message for α subunit have been found (Hartmann & Claudio, 1990; *see also* Buller & White, 1990*b*), and examination of oocyte mRNA by polymerase chain reaction amplification has indicated that at least some oocytes may contain appreciable levels of mRNA coding for γ subunit (C.F. Stevens, *personal communication*).

The conclusion to be reached is that *Xenopus* oocytes injected with sequences for all normal subunits express AChR with largely normal physiological properties. This is a minimal condition necessary for experiments involving expression of AChR with altered subunit stoichiometry or containing mutated subunits. Meeting this condition suggests that changes in functional properties seen in studies of structurally modified receptors can be interpreted as resulting from the structural change, rather than from the expression system.

D. Receptors Missing Subunits or Having Subunits from Several Species

Two main classes of receptor with altered subunit composition have been studied: receptors with one or more subunits missing and receptors composed of subunits from different species. The major motivation for these experiments has been to provide a coarse screen to identify subunits or regions of subunits which are likely to be important in determining gating properties of AChRs. Data which suggested that the δ subunit might play a major role in determining gating (Sakmann et al., 1985) was very influential in suggesting that this approach would be productive. At present, however, it appears likely that each subunit has effects on gating and attempts to identify a region of particular importance for gating have not been successful.

A four letter shorthand will be used in both of these sections; most experiments used α, β, γ and δ subunits from one or more species. The subunit composition will be designated by a one-letter code for the species of origin for the subunit (M = mouse, B = bovine, T = *Torpedo*, X = *Xenopus*). Examples are: MMMM (all four subunits from mouse), MM-M (mouse α, β, δ subunits, no exogenous γ), and TBTB (Torpedo α and γ with bovine β and δ).

Oocytes have been injected with mRNA for *Torpedo*, mouse or calf receptor subunits and comparisons made between the full and partial complements of subunits. The first point is that the omission of any one subunit reduces both the expression of surface αBGT-binding sites and the ACh-elicited current (Table 3). Expression of surface receptors when assayed by αBGT-binding is higher with αβγ than with αβδ subunits (Kurosaki et al., 1987; Sumikawa & Miledi, 1989), whereas, in general, more ACh-elicited current is found with αβδ subunit injections (Kurosaki et al., 1987; Mayne et al., 1987; Jackson et al., 1990; Lo et al., 1990*a*). The divergence between surface expression and cholinergic current probably reflects a lower single channel conductance and a stronger voltage dependence of gating for αβγ (White, 1987; Kullberg et al., 1990).

Table 3. Effect of missing subunits on expression

AChR[a]	pS/site	nA	ACh (μM)	Notes
TTTT	2×10^{-4}	132	1	1
TTT-	6×10^{-5}	13	1	1
TT-T	2×10^{-5}	0.4	1	1
T-TT	4×10^{-6}	0.05	1	1
TTTT	2×10^{-3}	600	1	2
TTT-	3×10^{-4}	17	1	2
BBBB	ND	1600	0.1	3
BB--	ND	<2	10	3
B--B	ND	<2	10	3
BB-B	ND	1000	0.01	3
BBB-	ND	130	1	3
MMMM	ND	1300	10	4
MM-M	ND	400	10	4
MMM-	ND	100	10	4

Notes:
[1] Kurosaki et al., 1987. Whole oocyte responses at −60 mV, *RT*. All other combinations were tested, with no detectable responses.
[2] Mayne et al., 1987. −60 mV, *RT*.
[3] Jackson et al., 1990. −70 mV, *RT*.
[4] Lo et al., 1990*a*. −30 mV, *RT*.
ND: not determined.
[a] Species of origin given in order of subunits (α, β, γ, δ) as T. Torpedo, B: Bos, M: Mus, X: Xenopus, −: omitted.

A confusing feature of the literature is that some authors apparently have greater success with particular subunit combinations than others; why is not clear and quantitative comparisons of protein synthesis and surface expression of assembled AChRs are rare.

There have been three studies of single channel currents for AChRs assembled in the absence of one subunit: BB-B (Jackson et al., 1990), MM-M (Lo et al., 1990*a*), and MMM- (Kullberg et al., 1990). The bovine and mouse "γ-minus" receptors gave different results (no change in burst duration for MM-M but a large increase for BB-B; *see* Table 4*B*). A second difference is that a high frequency of "spontaneous openings" (defined as openings in the absence of any added agonist) occurs with BB-B AChRs, but apparently not with MM-M AChRs. The mouse MMM- AChR has a burst duration about one-half that of control at −200 mV (the only potential reported; Kullberg et al., 1990).

It is surprising that functional AChRs can assemble when one subunit is not expressed. The most likely reason that responses can be generated when either the γ or the δ subunit is omitted is that the γ and δ subunits may substitute for each other,

Table 4. Effect of subunit composition on burst durations

A. Chimera	Mean burst duration (msec)	(N)	Notes
TTTT	0.3 ± 0.03	(11)	1
TMTM	0.4 ± 0.07	(9)	1
MTTT	3.3 ± 0.2	(5)	1
MMTM	3.9 ± 0.4	(5)	1
TTTM	3.9 ± 0.6	(5)	1
MMXM	5.9	—	2
MMMM	8.4 ± 1.9	(9)	1
MTMM	18.3 ± 3.5	(4)	1
TTTT	0.6 ± 0.2	(3)	3
BTTT	2.5 ± 0.6	(4)	3
BBBB	7.6 ± 1.4	(4)	3
TTTB	8.6 ± 1.3	(3)	3

B. Missing subunits	Mean burst duration (msec)	(N)	Notes
BBBB	10.2 ± 1.4	(7)	4
BB-B	53 ± 22	(24)	5
MMMM	8.2 ± 0.5	(27)	6
MM-M	8.6 ± 1.2	(7)	6
MMMM	7.6 —	—	7
MM-M	7.6 —	—	7
MMMM	33	(5)	8
MMM-	11.3 ± 4	(3)	9

Notes:
[1] Yu et al., 1991. Mean burst duration with maximum closed time in burst of 0.1 msec, recorded from outside-out patches, −60 mV, 12°C, [ACh] 0.2–2 μM.
[2] Charnet et al., 1991. Mean burst duration corrected from value given at 0 to −60 mV; otherwise conditions as 1.
[3] Sakmann et al., 1985. Mean burst duration with maximum closed time of 1 msec, recorded from outside-out patches at −70 to −100 mV, 18–21°C, [ACh] 0.1–5 μM.
[4] Mishina et al., 1986. Mean burst duration with maximum closed duration in a burst of 1 msec; recorded cell attached at −70 to −100 mV, 18–20°C.
[5] Jackson et al., 1990. Conditions as 4, except at −90 mV.
[6] Lo et al., 1990*a*. Maximum closed time 0.1 msec, recorded outside-out at −70 mV, 20–22°C.
[7] Charnet et al., 1991. Maximum closed time 0.1 msec, recorded outside-out at 12°C, corrected to −70 mV from data provided for 0 mV.
[8] Kullberg et al., 1990. Maximum closed time 0.3 msec, recorded cell attached at 20–22°; value at −200 mV corrected from data provided for −100 mV.
[9] Kullberg et al., 1990. As for 8, but data obtained at −200 mV.

albeit inefficiently. One alternative explanation is that AChRs are assembled with endogenous *Xenopus* subunits produced by the oocyte. This is certainly conceivable, especially as only very limited data are available on the properties of AChRs expressed when *Xenopus* subunits are injected into oocytes (Charnet et al., 1991). However, endogenous subunits cannot provide a simple explanation for all the data (for instance why injection of α, β, γ *or* α, β, δ subunits produces AChRs but not injection of α, β, ε subunits; Jackson et al., 1990; Liu et al., 1990).

The idea that the subunit composition can have a major influence on gating is supported by the demonstration that fetal and adult receptors are distinguished by a single subunit change: adult receptors with $\alpha_2\beta\varepsilon\delta$ and fetal with $\alpha_2\beta\gamma\delta$ composition (Mishina et al., 1986). This observation was made after the first cross-species chimeric receptors were expressed (*see below*), and confirmed that subunits other than the ACh-binding (α) subunit can affect gating. The specific differences between the γ and ε subunits which underlie the differences in gating have not yet been identified, although it appears that the M1 and M2 regions are not crucial in this respect (Villarroel et al., 1991; *see below*).

A number of chimeric receptors have been studied in which a full complement of subunits are expressed, but subunits from different species are used. The mean burst duration and its voltage dependence for AChRs from different species can be quite different; most comparisons have been made between AChRs from *Torpedo* (which have a brief burst duration and low voltage dependence) and mammalian fetal receptors (whose burst duration is an order of magnitude longer and whose voltage dependence is at least fourfold larger). In the first experiments using cross-species chimeric receptors (Sakmann et al., 1985), properties of AChRs with various *Torpedo* and bovine subunits were compared. The results suggested that the δ subunit might be important for determining gating properties since TTTB receptors bore a closer resemblance to BBBB than to TTTT receptors (*see* Table 4*A*). A study of mouse/*Torpedo* hybrids indicate that the situation is not that simple; both β and δ subunits strongly influenced the voltage sensitivity (Yoshii et al., 1987). All combinations that include both mouse-β and *Torpedo*-δ have the highest sensitivity, and all combinations that include both *Torpedo*-β and mouse-δ have the lowest sensitivity. Analysis of single channel records (Yu et al., 1991) also showed no clear relationship of burst durations to subunit composition (Table 4*A*).

E. Mutations

1. Some Mutations in Membrane Spanning Regions Alter Function

A large number of mutant subunits has been made (more than 100 are mentioned in the literature; Tobimatsu et al., 1987; Mishina et al., 1985; Imoto et

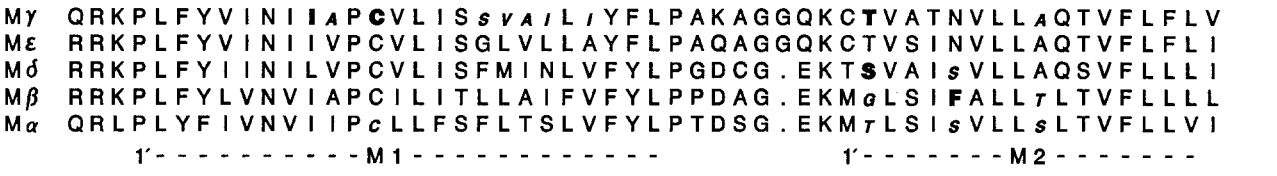

Fig. 3. Amino acid residues substituted in the M1-M2 regions. The amino acid sequences determined for mouse receptor subunits are shown using the single letter code, in the region containing the M1 and M2 membrane-spanning helices (indicated on bottom line). The numbering convention used for residues is to take the first position in the aligned regions to be position 1'. Hence, in the M2 region the mouse α subunit has a threonine (αT2'), whereas the δ subunit has a serine (δS2'). The dots in the sequences of α, β and δ subunits show a single location at which the γ and ε subunits apparently had an insertion. Residues shown in italic face have been substituted with no apparent change in kinetics; some mutations of residues shown in bold face have resulted in changes (*see text*). (The substitutions in the M2 region which had no effect were: αT2'A, αS6'A, αS10'A, βG2'S, βT10'A, γA10'S and δS6'A.)

al., 1986, 1988; Leonard et al., 1988*a*; Charnet et al., 1990; Lo et al., 1990*b*), but data on burst kinetics have only been reported for 16 and some data on whole oocyte responses for another 18. It is not clear why progress is slow, except that kinetic studies are time consuming.

Tobimatsu et al. (1987) studied the effects of swapping entire membrane-spanning regions of the α subunit. The most interesting result was that replacement of the M4 membrane-spanning helix with membrane-spanning helices from either of two unrelated proteins actually increased the ACh-elicited conductance per surface αBGT-binding site by an order of magnitude. A similar increase occurred when the M4 region was truncated by four residues. These observations are surprising since exchanges of M1, M2 or M3 regions resulted in very low expression of surface αBGT-binding sites and low conductance per site. Unfortunately, no single channel data on these mutant receptors have been reported, so it is not clear whether the single channel conductance or activation properties are affected. The cysteine residue at position 451 of the *Torpedo* γ subunit (close to the N-terminal or cytoplasmic end of M4) has been mutated to serine or tryptophan (Li et al., 1990). Both changes resulted in expression of a normal number of surface αBGT-binding sites, but reduced the conductance per site by about 50%. Again, it is not clear how to interpret the reduction. An earlier study (Pradier et al., 1989) had expressed receptors with mutations of cysteine residues in an amphipathic region slightly towards the N-terminus of M4 in the γ subunit (position 416 and 420), which had much less effect on ACh-elicited current.

Only one group has specifically set out to examine the effects of multiple substitutions of a single residue on channel gating. The residue is a conserved cysteine, twelve amino acids from the N-terminal end of the M1 membrane spanning region (see Figs. 2*C* and 3; Lo et al., 1990*b*). This position was mutated to several other residues in the γ subunit, with one mutation made in the α subunit as well. Changes in the γ subunit produced changes in burst duration which correlated with the change in volume of the side chain: when side chain volume was reduced (cys- > gly, ala or ser) the open duration was reduced, whereas a small increase in volume (cys- > thr) produced a slight lengthening (*see* Table 5). Surprisingly, substitution

Table 5. Effect of point mutants on burst durations

Mutant	Mean Burst Duration (msec)	(N)	Notes
Substitutions in M1 region[a]			
MMMM	8.2 ± 0.5	(27)	1
γC12'G	2.9 ± 0.3	(4)	2
γC12'A	5.4 ± 0.2	(2)	2
γC12'S	5.5 ± 0.7	(6)	2
γC12'T	9.3 ± 1.6	(2)	2
γI9'V	13.9 ± 2.3	(5)	1
αC12'S	8.4 ± 0.5	(5)	2
Substitutions in M2 region[b]			
MMMM	15.3 ± 1.2	(4)	3
βF6'S	5.0 ± 1.0	(3)	3
δS2'A	29 ± 9	(2)	3

Notes:
[1] Lo et al., 1990*a*. Mean burst duration with maximum closed duration in a burst of 0.1 msec, recorded from outside-out patches, −70°C, 20–24°.
[2] Lo et al., 1990*b*. Conditions as 1.
[3] Charnet et al., 1990. Mean burst durations with maximum closed duration of 0.2 msec recorded from outside-out patches, −150 mV, 12°C.
[a] Mouse subunits were expressed in *Xenopus* oocytes. Either all normal subunits were injected (MMMM), or three normal subunits were injected plus a single mutated sequence. The mutation is indicated by the subunit, the original residue, its location in the M1 region, and the altered residue (e.g., in the γ subunit the cys residue at position 12' of the M1 region was replaced by gly). For location of residues, *see* Figs. 2 and 3.
[b] Mutants are designated in an analogous manner to those in the M1 region, except in this case substitutions were made in the M2 region.

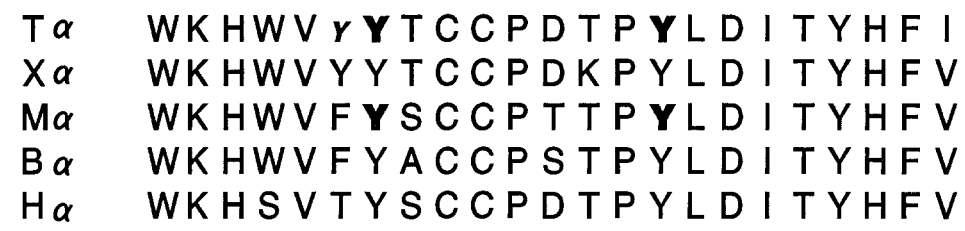

Fig. 4. Amino acid residues substituted near the ACh-binding site. The amino acid sequences around residues 192 and 193 are shown for *Torpedo californica* (*T*), *Xenopus* (*X*), mouse (*M*), bovine (*B*) and human (*H*) α subunits. The tyrosine residues in bold face (Y^{190} and Y^{198}) have been mutated to phenylalanine in *Torpedo* and mouse subunits with marked reductions in ACh-binding affinity. Tyrosine-189 in the *Torpedo* sequence has been mutated to phenylalanine (as occurs in the mouse sequence normally) with no effect on binding.

of the homologous cysteine in the α subunit by serine had no effect. The data obtained with the γ subunit have been interpreted in terms of a simple physical model in which increasing the volume of an uncharged side chain makes the energy barrier higher for the channel closing reaction. The data demonstrate that this residue of the M1 region of the γ subunit has an effect on channel gating. However, the model cannot give a simple account of differences between the γ and ε subunits, as both have the conserved cysteine residue. Indeed, in a recent study each residue which differs between the γ and ε subunits in the M1 region was switched in turn, and it was found that none of the switches had any effect on channel burst duration (Villarroel et al., 1991).

Interest in the M2 region has focused on questions of ion permeation and channel block. Burst kinetics have not been reported for many of the mutants prepared, including all of the mutants of ionizable groups (Imoto et al., 1986, 1988). Study of local anesthetic block required analyses of burst kinetics, and so data are available on kinetics after changes in the polarity of equivalent residues in four mouse subunits (α, β, γ, δ; Leonard et al., 1988*a*; Charnet et al., 1990; the residues are indicated in Fig. 3). Mutations in two residues produced a lengthening of about 60% of the open time and burst duration (thr- > ala at position 2′ of γ and ser- > ala at the equivalent position of δ); when both mutant subunits were expressed together the burst duration was doubled. Each of these mutations involves a reduction of side-chain volume, as well as a reduction in polarity. On the other hand, mutation of a residue in the β subunit (phe- > ser at position 6′, one full turn along the helix towards the external surface) reduced the burst duration to about 30% of control. This mutation would result in a volume decrease as well. Seven other mutations at position 2′, 6′ or 10′ in the M2 region had no clear effect on burst duration (legend to Fig. 3).

2. Mutations Near the ACh-Binding Site Change Binding

Biochemical studies in which receptors have been covalently labeled indicate that at least part of the ACh-binding site is located in a region of the α subunit immediately around a pair of cysteine residues at positions 192 and 193 (*see* Figs. 2*C* and 4). Recent work with point mutations in this region has shown that the nearby tyrosine residues (at positions 190 and 198) are important in determining the affinity for agonists. These residues are conserved in all α subunits and have been substituted in α subunits from *Torpedo* (O'Leary & White, 1991) and mouse (Tomaselli et al., 1991). When mutated α subunit is expressed with normal β, γ, δ subunits, alteration of tyrosine-198 to phenylalanine changes the EC_{50} for ACh-elicited currents from 23 to 115 μM (*Torpedo*) or 10 to 60 μM (mouse), whereas tyrosine-190 to phenylalanine changes the EC_{50} to 430 μM (*Torpedo*) or 500 μM (mouse). For the mutated mouse subunits the ACh affinity also has been measured from inhibition of αBGT-binding, with parallel results. It might be expected that reduced affinity would involve an increase in the dissociation rate, and hence the burst duration and number of openings per burst might decrease. However, no kinetic analyses have yet been performed.

F. Channel Gating May Involve the Concerted Participation of Multiple Domains on Multiple Subunits

What have we learned from omissions or swaps of entire subunits? The muscle AChR is able to assemble and function despite having an abnormal complement of subunits. It had been hoped that it would be possible to identify subunits which showed strong effects on gating which would indicate those sequences which should be explored further. This hope has not been realized, largely because the data

suggest that each subunit can affect gating. Moreover, it seems that subunits other than the α subunit can affect the affinity for ligands.

The use of mutated subunits is potentially more discriminating, since it is more likely that most of a mutated subunit will maintain normal interactions with the other subunits in the assembled receptor and hence that the effects of the mutation would be more interpretable. Unfortunately, few kinetic data have been reported for mutants. One interesting result is that substitution of the residues which differ between γ and ε subunits in the M1 and M2 regions does not convert the channel open time (Villarroel et al., 1991). The data suggest that at least some residues in the putative membrane-spanning regions M1, M2 and M4 can influence gating (M3 has not been studied), in agreement with the prejudice that channel gating involves rearrangements in transmembrane structures. Although these data conform to what might be expected, it is also interesting that a number of mutations in these regions did *not* have any measurable effect on gating.

An approach that was used with great success to identify regions important in ion permeation was the exchange of larger regions of γ and δ subunits (Imoto et al., 1986) followed by substitutions of selected residues (Imoto et al., 1988). No data on channel kinetics from such "medium grained" exchanges (for example, between γ and ε) have been reported as yet.

In summary, the data support the idea that gating is a distributed property of the entire AChR pentamer: all subunits are instrumental in determining gating properties. The data also support a conclusion of high structural specificity in gating, as identical mutations of homologous residues in different subunits can have quite divergent effects on gating. Most likely, gating is determined by critical residues in specific subunits, but the subunit interactions thought to occur during gating dictate that the effects of a given mutation will depend on the background of the other subunits. This statement is in agreement with data from other multimeric proteins (e.g., Perutz, 1990), and simply says that the trigger is larger than we might have naively hoped.

V. Summary and Conclusions

Work over the past ten years has greatly increased our understanding of both the structure and function of the muscle nicotinic acetylcholine receptor. There is a strongly supported general picture of how the receptor functions: agonist binds rapidly to sites of low affinity and channel opening occurs at a rate comparable to the agonist dissociation rate. Channel closing is slow, so the channel has a high probability of being open if both agonist-binding sites are occupied by ACh. Results of expression studies have shown that each subunit can influence AChR activation and have given a structural basis for the major physiological change known for muscle AChR, the developmental change in AChR activation. These general statements notwithstanding, there are still major areas of uncertainty which limit our understanding. We have emphasized these areas of uncertainty in this review, to indicate what needs to be done.

First, the quantitative estimates of rate constants are not as strongly supported as they should be. The major reasons are twofold—uncertainties about the interpretation of components in the kinetic data and difficulties of resolving brief events. As a result, any inferences about the functional consequences of structural alterations must remain tenuous.

Second, the functional behavior of individual AChRs is not as well understood as it should be. The kinetic behavior of an individual receptor clearly can be complex (section II). In addition, there is evidence that superimposed on this complexity there may be stable and kinetically distinguishable populations of receptors (section III). Until the basis for the kinetically defined populations is clarified, kinetic parameters for receptors of defined structure cannot be unambiguously obtained.

Finally, it is not surprising that the studies of AChR of altered structure have not given definitive results. Two reasons should be apparent from the preceding points: there is not a fully supported approach for kinetic analysis, and the "normal" population may not be clearly defined. An additional complication is also emerging, in that the available data support the idea that specific residues distributed over all subunits may influence AChR activation. This possibility renders the task of analysis that much more difficult.

The muscle nicotinic AChR has served as a prototype for the family of transmitter-gated membrane channels, which includes the muscle and neuronal nicotinic receptors, the $GABA_A$, the glycine and possibly the non-NMDA excitatory amino acid receptor (Stroud et al., 1990). It is interesting to note that the functional properties of the $GABA_A$ receptor, probably the best-studied of the other members of the family are rather similar. In particular, open-time and burst durations show multiple components interpreted as reflecting openings of singly and doubly liganded receptors (Mathers & Wang, 1988; Macdonald et al., 1989), the distribution of gaps indicates a relatively complex gating scheme (Twyman et al., 1990; Weiss & Magleby, 1989), and mul-

tiple kinetic modes are likely to exist (Newland et al., 1991). The situation with regards to the effects of $GABA_A$ receptor subunit stoichiometry is more complex than for muscle AChR (e.g., Luddens & Wisden, 1991), perhaps similar to that found for neuronal nicotinic AChR (Papke et al., 1989; Luetje et al., 1990; Luetje & Patrick, 1991). Overall, it appears that the unresolved questions about the muscle nicotinic AChR are not indications that this is an exceptionally complicated transmitter-gated channel. Rather, it appears to be a relatively straightforward member of the family, and the lessons we learn from studying it are likely to be directly applicable to other receptors.

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