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DEPENDENCE OF ACETYLCHOLINE RECEPTOR CHANNEL KINETICS ON AGONIST CONCENTRATION IN CULTURED MOUSE MUSCLE FIBRES

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SUMMARY

1. The patch-clamp technique was used to study channel gating kinetics of the acetylcholine receptor. The agonist carbachol was used at concentrations varying from 0·10 to 20 μM.

2. Data in which many channels were often open at the same time were analysed with the aid of mathematical expressions that relate the stochastic behaviour of a many-channel system to the kinetic parameters of a single channel. These methods provide consistent estimates of parameters. This consistency suggests that there is no correlation between the kinetics of channel closure and the density of channels in a patch of membrane.

3. Closed times were well fitted by a sum of two exponentials. Addition of a third exponential component never significantly improved the quality of the fit.

4. A sum of two exponentials usually provided the best fit to open times. The ratio of the fractions of slowly closing and rapidly closing channels increased linearly with agonist concentration, in a manner consistent with the opening of singly and doubly liganded receptor channels.

5. Analysis of closed-time densities at various times after seal formation was used to follow the time course of desensitization. No changes in the kinetics of channel closure were detected during desensitization.

6. At 0·10 μM-carbachol the frequency at which openings were observed was only slightly more than the background frequency of spontaneous opening. At 20 μM-carbachol, immediately after seal formation and before the onset of desensitization, the frequency of opening was approximately 300 times higher.

7. The frequency of appearance of brief-duration openings increased linearly with carbachol concentration and saturated at approximately 5 μM. The frequency of appearance of long-duration openings increased as the square of the agonist concentration, with only a slight hint of saturation.

8. The results presented here are discussed within the framework of a two-binding-site model for the allosteric activation of the acetylcholine receptor. Estimates are made of all of the equilibrium constants and many of the rate constants of the relevant reaction scheme. The two ligand binding sites are found to be very different in terms of their dissociation constants and their influence on the channel gating

transitions. These results have implications for the energetics of receptor activation and for the utilization of binding energy by the receptor.

INTRODUCTION

Elucidation of the mechanism of activation of the nicotinic receptor by acetylcholine is essential to the understanding of synaptic transmission at the neuromuscular junction. With the general acceptance of the neuromuscular junction as an important model for synaptic transmission, the nicotinic receptor holds an important place as a model for a class of synaptic receptors that are physically associated with, and directly coupled to, an ion channel. More generally, the nicotonic receptor is useful as a model for allosteric proteins. Thus, we have compelling reasons for asking how the transmembrane channel of the acetylcholine receptor is activated by the binding of ligand.

The study of response as a function of agonist concentration is a direct and very widely employed strategy for the study of receptor activation (Colquhoun, 1973). The power of this approach stems from the quantitative predictions that are made by applying the basic principles of chemical equilibria and kinetics to different reaction schemes (Monod, Wyman & Changeux, 1965; Koshland, Nemethy & Filmer, 1966; Eigen, 1967; Fersht, 1985). This kind of analysis of macroscopic membrane currents has yielded evidence that maximal activation of the acetylcholine receptor occurs when two ligand binding sites are occupied (Katz & Thesleff, 1957; Adams, 1975; Land, Podleski, Salpeter & Salpeter, 1977; Dionne, Steinbach & Stevens, 1978). One of these studies (Dionne *et al.* 1978) has suggested that singly liganded receptors make a small but detectable contribution to the response at low agonist concentration.

Studies of the effect of ligand concentration have also been carried out with the patch clamp at the single-channel level. Some of these studies suggest that singly liganded receptor channels can open infrequently, and when they do open, they close more rapidly than doubly liganded receptor channels. The longer duration doubly liganded receptor channel openings predominate at higher ligand concentrations (Takeda & Trautmann, 1984; Colquhoun & Sakmann, 1985; Labarca, Montal, Lindstrom & Montal, 1985; Auerbach & Lingle, 1986). However, there is no consensus on these points: in some studies the requisite increase with concentration of long-duration openings was not observed (Morris, Wong, Jackson & Lecar, 1983; Sine & Steinbach, 1984*a*). There is a great incentive to resolve this issue. If singly liganded receptor channel openings can be detected and distinguished from doubly liganded receptor channel openings, then the opportunity exists to quantify the various components of the activation process in a manner which has not yet been achieved for any allosteric protein.

Previous studies at the single-channel level have been confined to experimental conditions under which the channel opening frequency is low enough to apply a stochastic theory for a system with only one channel (Colquhoun & Hawkes, 1981, 1982). This theory is applicable when channel openings are well separated and overlap rarely. To obtain such conditions, data are collected either at low agonist concentrations or from patches of membrane with low channel density. Alternatively,

investigators have taken advantage of desensitization by waiting for a loss of activity to occur (Sine & Steinbach, 1984*a*).

The stochastic theory of channel gating has recently been extended to a system with an arbitrary number of channels (Jackson, 1985). With the aid of this theory, current records with a high frequency of channel opening have been analysed. This has made it possible to make a detailed analysis of channel gating, before the onset of desensitization, for agonist concentrations varying over a 200-fold range.

METHODS

Cultured muscle fibres

Thigh muscle was removed from 1-day-prenatal mouse embryos, newborns, or 1-day-old pups. The tissue was minced, treated with 0·25% trypsin at 36 °C for 30 min, and triturated. Cells were isolated by repeated trituration and centrifugation, and were then plated on Primaria culture dishes (Falcon) at a density of 3×10^5 cells per 35 mm dish. Ham's F-12 culture medium was supplemented with 10% each of horse serum and fetal calf serum. The medium was changed the first day. When the background cells became confluent and the myoblasts began to fuse (usually within 2–5 days) the medium was changed to a medium with 10% horse serum, 10 μM-fluorodeoxyuridine, and 50 μM-uracil in order to kill mitotic cells. Two days later the medium was replaced with medium supplemented with 10% horse serum and 1 μM-tetrodotoxin. Virtually all recordings were made on 7-day-old cultures to minimize the effect of variation of culture age. At this age cultures had numerous large, multinucleated, striated muscle fibres.

Single-channel recording

Single-channel currents were recorded with an EPC-7 patch-clamp amplifier (List Electronics/Medical Systems, Great Neck, NY, U.S.A.) by standard techniques (Hamill, Marty, Neher, Sakmann, & Sigworth, 1981). Glass capillaries had outer diameters of 1·2 mm and were purchased from Garner Glass Co. (Claremont, CA, U.S.A.). Patch electrodes were fabricated from either thin-walled aluminasilicate glass (inner diameter 0·9 mm) or thick-walled borosilicate glass (inner diameter 0·6 mm). The patch electrodes were coated with Q-dope to reduce their capacitance. Aluminasilicate electrodes had resistances of from 1·5 to 3·5 MΩ and formed seals of 4 GΩ or higher. Borosilicate electrodes had resistances of 10–20 MΩ and formed seals of 10 GΩ or higher. The patch clamp was always held at +50 mV in on-cell recordings. Assuming that a cell has a resting potential of −60 mV, the potential difference across the sealed-off patch is approximately −110 mV. Membrane current was filtered at 5 kHz and sampled at 40 kHz with an A–D converter (Model DT2782-A, Data Translation, Maynard, MA, U.S.A.) into an LSI 11/23 computer (Andromeda Systems, Canoga Park, CA, U.S.A.). Data sampling was continuous to a disc. The maximum possible sampling time was slightly more than 2 min at 40 kHz with this 10 Mbyte disc, but in general 20–30 s of sampling was sufficient to record a minimum of 200 channel opening events necessary for curve fitting. Data records that contained high noise levels or extraneous signals were thrown out.

All recordings were made at room temperature (21–24 °C). Cells were bathed in 140 mM-NaCl, 3 mM-KCl, 1 mM-$CaCl_2$, 1 mM-$MgCl_2$, 1 μM-tetrodotoxin, and 10 mM-glucose, buffered at pH 7·4 with 10 mM-HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid). Patch electrodes were filled with the same solution plus the appropriate concentration of carbachol, minus glucose and tetrodotoxin. Tetrodotoxin was needed in the bath to prevent spontaneous contractions, but tetrodotoxin was not needed in the patch electrode, because at −110 mV sodium channels did not open.

Data analysis

Channel current records were analysed in three stages. (1) Raw data sets were used to compute lists of closed times and isolated open times. (2) Closed times were fitted to sums of exponentials. (3) Isolated single-channel open times were curve fitted to probability density functions valid for a system with many channels (Jackson, 1985).

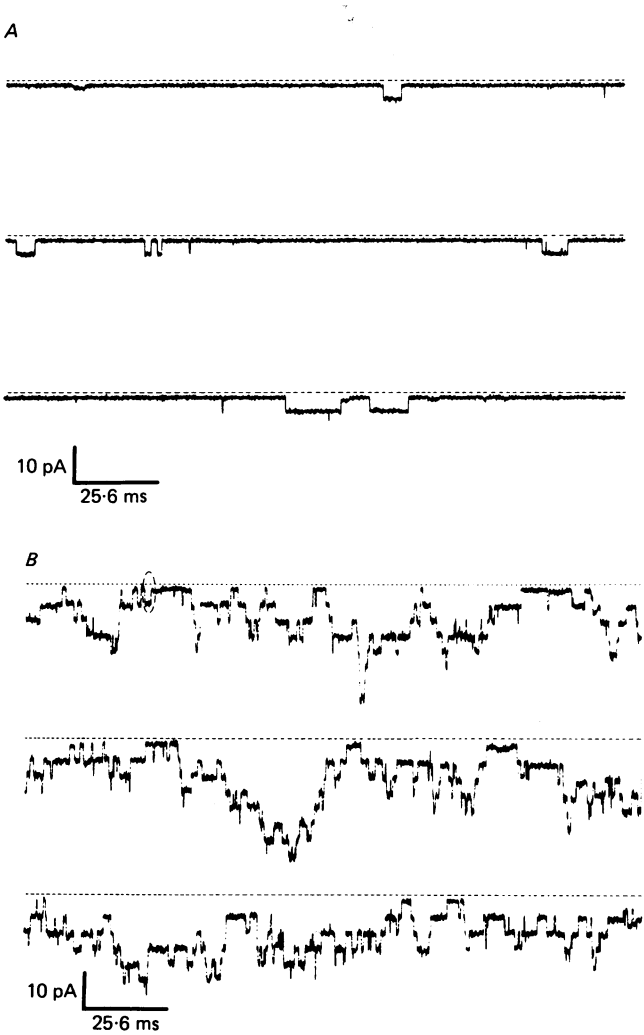

Fig. 1. Patch-clamp current data obtained from two patches with different levels of channel activity. Recordings were made with thick-walled borosilicate patch electrodes filled with 2 μM-carbachol. All but one of the single-channel currents in *A* are isolated, i.e. they are flanked on both sides by intervals with no open channels. In *B* there are many multiple-channel openings. The baseline currents are indicated with dashed lines just above each trace. Isolated single-channel currents are less common in *B*. An isolated single-channel current is circled in the top row of *B*. The frequencies of channel opening, λ_2, from the best-fitting closed-time distribution, were 0·03 ms^{-1} in *A* and 0·26 ms^{-1} in *B*.

The first stage of analysis was carried out with the aid of an LSI 11/23 computer. A program scanned digitized data sets point by point. When three successive points deviated by more than 1·4 pA from the average computed for up to twenty previous points since the last transition, or when the differences between three successive pairs of points were greater than 0·6 pA, a transition was recorded. With the 4–5 pA channel currents observed here, these criteria detected transitions with a very high efficiency, while false transitions were easily identified and removed. The program produced, as an output to a file, the current and time since the last transition. This fully automated program produced an output that was indistinguishable from that of a semi-automated program,

which allows the operator to evaluate each transition. Parameters obtained in curve fits to the same data, analysed in either a fully automated or in a semi-automated fashion, were indistinguishable.

The amplitude distributions of these output files had sharp peaks at the baseline current where all channels were presumably closed. Sharp peaks also appeared at integral multiples of the single-channel current. All intervals within two standard deviations of the baseline were put into a list of closed times. In general, there was no ambiguity about the baseline current. The baseline was always the highest current in the data record, and was consistently between −2 and 1 pA from one experiment to the next. Thus, intervals of time in the region of the baseline were times during which all acetylcholine receptor channels in the patch of membrane were closed. There was no evidence in the amplitude distribution for multiple unit conductances.

Lists of isolated openings were prepared by including intervals within two standard deviations of the first peak below the baseline, provided that these intervals were flanked on either side by closed times. In patches with low levels of channel activity (Fig. 1*A*), most channel openings were isolated. In patches with high levels of channel activity (Fig. 1*B*), there were many multiple openings, and isolated openings were less common. A typical isolated single-channel current is circled in the first row of Fig. 1*B*.

The second stage of analysis was the fitting of closed times to an appropriate probability density function. The closed-time density of a single Markov channel with n closed states is a sum of n exponentials of the form $p_1 = \sum_i^n w_i \omega_i e^{-\omega_i t}$ (Colquhoun & Hawkes, 1981), where ω_i are exponential decay constants and w_i are the weights of each exponential component. The closed-time density of a system of N identical channels, each with n closed states, has the form

$$p_{Nc}(t) = \frac{w_i \omega_i e^{-\omega_i t}\left[\sum_i^n w_i e^{-\omega_i t}/\omega_i\right]^{N-1} + (N-1)\left[\sum_i^n w_i e^{-\omega_i t}\right]^{-2}\left[\sum_i^n w_i e^{-\omega_i t}/\omega_i\right]^{N-2}}{\left[\sum_i^n w_i/\omega_i\right]^{N-1}}, \quad (1)$$

derived from relations 2–5 of Jackson (1985). When expanded, this expression is a sum of exponentials, justifying the use of an expression of the form

$$p_c(t) = \sum_i^r x_i \lambda_i e^{-\lambda_i t} \Big/ \sum_i^r x_i e^{-\lambda_i t_c}, \quad (2)$$

as a probability density function, where r, the number of exponential terms that can be detected in real data, is not readily inferred from eqn (1).

Lists of closed times were used to calculate a likelihood function with eqn (2). Likelihood maximization (Horn & Lange, 1983; Colquhoun & Sigworth, 1983) was then carried out on an IBM AT computer with a minimization program written by Dr Kenneth Lange and described previously (Jackson, 1986*a*). The parameters λ_i and x_i were varied under the constraint that the x_i add up to one. The factor in the denominator of eqn (2) effects a correction for missed brief openings, as described by Colquhoun & Sigworth (1983) (eqn (60) of this reference). A short time cut-off, t_c, of 0·0875 ms was selected because the sampling interval was 0·025 ms, and because intervals of less than four points could not be reliably detected. Since data were sampled continuously, no long-time cut-off was used.

It should be noted that the parameters in eqn (2), λ_i and x_i, could be quite different from the parameters ω_i and w_i (eqn (1)), relevant to the gating of a single channel. λ_i and x_i in eqn (2) should be considered empirical quantities, useful in quantifying the distribution of times during which all channels are closed. The decay constants, λ_i, of the sum of exponentials fit to the closed times can be thought of as phenomenological rate constants of channel opening. The relationship between these quantities and the actual single-channel rate constants will be dealt with in the Discussion. Regardless of this relationship, eqn (2) is essential in the subsequent analysis of open times, as described next.

In the third stage of analysis, lists of isolated open times were fitted to the expression

$$p_o'(t) = p_o(t) \sum_i^r (x_i/\lambda_i) e^{-\lambda_i t} \Big/ \int_{t_c}^{\infty} p_o(t') \sum_i^r (x_i/\lambda_i) e^{-\lambda_i t'} dt', \quad (3)$$

(Jackson, 1985), again using likelihood maximization. The denominator is the correction for missed brief events similar to that made in eqn (2) (Colquhoun & Sigworth, 1983). Equation (3) is not identical to the original equation derived previously (Jackson, 1985), but includes an approximation of $(N-1)/N \simeq 1$. This approximation is justified because N, the number of channels, is large. In general, the fitting is carried out without knowing N. This quantity is estimated in the Discussion. The parameters λ_i and x_i in eqn (3) are taken from a prior fit of eqn (2) to closed times from the same data. p_o, the open-time probability density for a single channel with m closed states, is

$$p_o(t) = \sum_i^m \nu_i y_i e^{-\nu_i t}. \quad (4)$$

When eqn (3) is fitted to isolated open times, only y_i and ν_i are varied. Equation 3 corrects the single-channel open-time density (eqn (4)) for openings of other channels. In patches with more channel activity, a long-duration opening is more likely to be interrupted by the opening of other channels. The function of closed-time parameters, included as part of eqn (3), takes this into account, reducing the probability of long-duration openings.

In contrast to the parameters obtained in fits of closed times, the parameters ν_i and y_i, obtained by fitting eqn (3), are not empirical, but are related in a model-dependent fashion to the rate constants that operate in the gating of a single channel. Evidence for the validity of parameters estimated with eqn (3) will be presented in Results.

Curve fits were carried out with different numbers of exponential terms, and different models were compared with the likelihood ratio test (Rao, 1973). Except where noted, the minimization process yielded error estimates for each curve fit that were generally comparable to the standard deviations of the parameters determined from several curve fits to similar data sets.

RESULTS

The kinetics of channel opening

The kinetics of channel opening were studied by analysing the distributions of time intervals during which all channels were closed. Lists of closed times were fitted to sums of exponentials with different numbers of terms (eqn (2)), as described in Methods. The results of these fits were then examined.

Closed times were well fitted by a sum of two exponentials. In no instance did the addition of a third exponential component result in a statistically significant improvement (at the 5% level). Figure 2*D* and *E* displays plots of closed-time densities with best-fitting sums of two exponentials drawn for comparison. Some current records provided lists of 3000 or more closed times, but most lists included from 200 to 500. In many of these data sets the addition of a second exponential component resulted in a very large increase in the maximum likelihood and consequently produced a very high χ^2 (as high as 160). Thus, the failure of a third exponential to increase the maximum likelihood is a clear indication that a third component, if it exists, should be very small.

For carbachol concentrations of 1 μM or less, a second exponential often did not significantly improve the fit to closed times. In these data sets (not shown) only a single slow exponential component could be detected. x_1, the fraction of brief closures, was assumed to be zero in these data sets, since a failure to detect the fast component of closures suggests that if it is present, its weight is very low. In other data sets obtained with low carbachol concentration a second component of brief closures was evident (Fig. 2*D*). At 20 μM, and to a lesser extent 10 μM-carbachol, most closed times were so brief that it was difficult to detect a second exponential

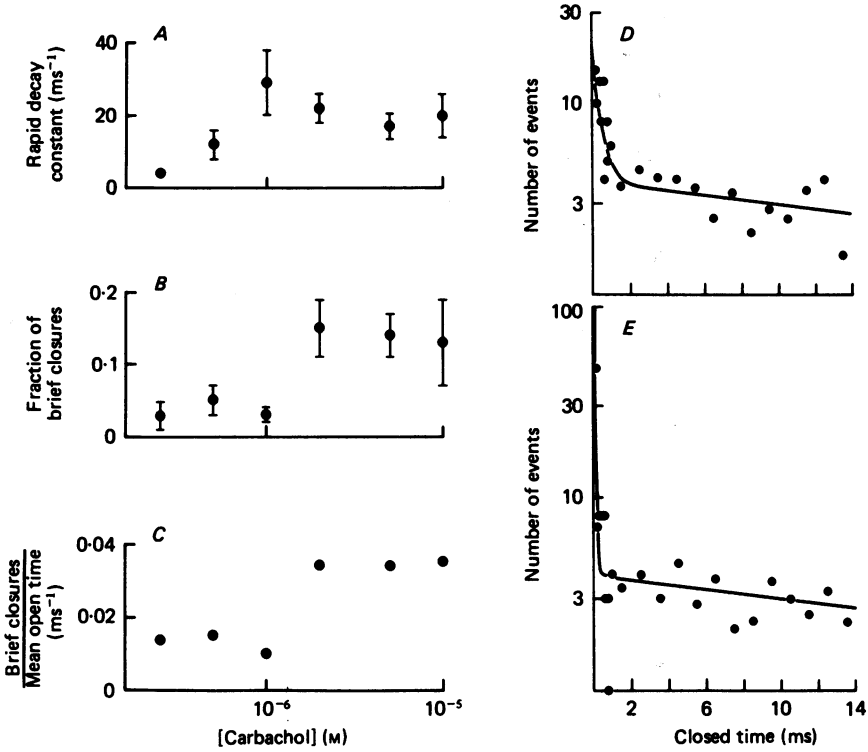

Fig. 2. The fast decay constant, λ_1 (*A*), and the fraction of brief closures, x_1 (*B*), obtained by fitting a sum of two exponentials (see eqn (2) in Methods) to closed times, are plotted *versus* the logarithim of the carbachol concentration. Standard errors are also indicated. From six to fifteen data sets (an average of eleven) were used for each concentration. *C* displays the fraction of brief closures divided by the mean open time. *D* and *E* are representative closed-time densities (number of events per 100 μs) at 0·5 and 5·0 μM-carbachol respectively. The best-fitting sum of two exponentials is drawn as a smooth curve. In these density plots bins are 0·1 ms from 0 to 1 ms and 1 ms above 1 ms, with the numbers in the long time bins scaled by a factor of ten. The best-fitting parameters were $\lambda_1 = 2{\cdot}5$ ms^{-1}, $x_1 = 0{\cdot}04$, and $\lambda_2 = 0{\cdot}028$ ms^{-1} in *D*, and $\lambda_1 = 27$ ms^{-1}, $x_1 = 0{\cdot}25$, and $\lambda_2 = 0{\cdot}027$ ms^{-1} in *E*.

component. Between 2 and 10 μM-carbachol a distinct component of very brief closures was usually observed (Fig. 2*E*) with an average decay constant of 22 ms^{-1}.

The fast component of closures was at the limits of time resolution when the bandwidth was set at 5 kHz. Four experiments were carried out at 10 kHz with 2 μM-carbachol, and data were sampled in discrete records at 125 kHz. The value of the fast decay constant obtained from these data was 26 ms^{-1}, suggesting that using a bandwidth of 5 kHz (the maximum continuous sampling frequency of this system) does not produce a large error in the measurement of this decay constant.

The kinetic parameters obtained from closed times at each concentration of carbachol were averaged and are displayed in Figs 2*A–C* and 3. Between six and fifteen data sets were used for each concentration of carbachol. In Fig. 2*A* and *B* the fast decay constant in the closed-time distribution, λ_1, and the weight of this fast

component, x_1, are displayed. It appears that λ_1 increases with concentration, reaching a plateau of approximately 22 ms^{-1} at 1 μM. However, the mean values of λ_1 at low carbachol concentrations are difficult to interpret, since the number of brief closures is so low. Thus, no conclusion can be reached about whether λ_1 is a meaningful quantity at low agonist concentration.

The fraction of brief closures switches abruptly from around 0·03 to 0·14 (Fig. 2*B*). A *t* test shows that all of the values 1 μM or below are significantly different from those above 1 μM, except for the value at 10 μM, where the error is higher because there is some difficulty in resolving the two components of closures. The plateau in the decay constant of the brief closures, and the transition in the fraction, both appear near 1 μM. Open time also increases with carbachol concentration (data presented below). It is possible that there is a constant frequency of appearance of brief closures per unit of time spent in the open state; the increase in brief closures would then be a secondary consequence of an increase in open time. To test this hypothesis the fraction of brief closures was divided by the mean open time computed for that concentration to give a fraction of brief closures per unit time in the open state. The plot of this ratio (Fig. 2*C*) shows the same trend seen with the fraction of brief closures (Fig. 2*B*), indicating that the increase in brief closures is an increase in the frequency of such events per unit of time in the open state.

The relatively low fraction of brief closures ($x_1 \simeq 0{\cdot}15$) means that, provided that there are no additional undetected components, nearly all closed times are resolved in these data. This obviated the need to analyse bursts of openings, which depend on many more kinetic parameters than open times. Errors in mean open times due to unresolved closures are 9% or less.

Variation in channel opening frequency

The slowest decay constant from the closed-time distribution, λ_2, increases with concentration (Fig. 3). The magnitude and monotonicity of this increase suggest that the underlying process is dependent on ligand binding. It is useful at this juncture to identify λ_2 as the apparent frequency of opening of channels in a patch. This will be discussed further below.

The frequency of opening is highly variable from patch to patch for a given concentration of carbachol. In contrast to the other parameters, the standard deviations of λ_2 from individual curve fits are much larger (by more than an order of magnitude) than the error estimates from each curve fit. This variability may reflect some intrinsic property of the muscle cell membrane. Since this rate process appears to be associated with receptor activation, it should be proportional to the number of channels in the patch. The number of channels in a patch could vary as a result of variations in the area of the patch of membrane or as a result of variations in the density of channels in the cell membrane.

Electrodes fabricated from aluminasilicate and thick-walled borosilicate glass have very different tip areas and shapes (Sakmann & Neher, 1983). With 0·5 μM-carbachol, the mean frequency of opening from eight experiments with alumina-silicate electrodes was 4·1-fold higher than the mean from six experiments with borosilicate electrodes. This indicates that patch area is an important quantity in determining opening frequency. This factor of 4·1 was used as an estimate of the ratio

of average tip areas of electrodes fabricated from these two glasses. Before plotting in Fig. 3, measurements of opening frequency made with aluminasilicate electrodes were corrected by the factor 4·1 to account for differences in tip area of electrodes made from the two kinds of glass. Only aluminasilicate electrodes were used below 0·5 μM-carbachol and only borosilicate electrodes were used above 0·5 μM-carbachol.

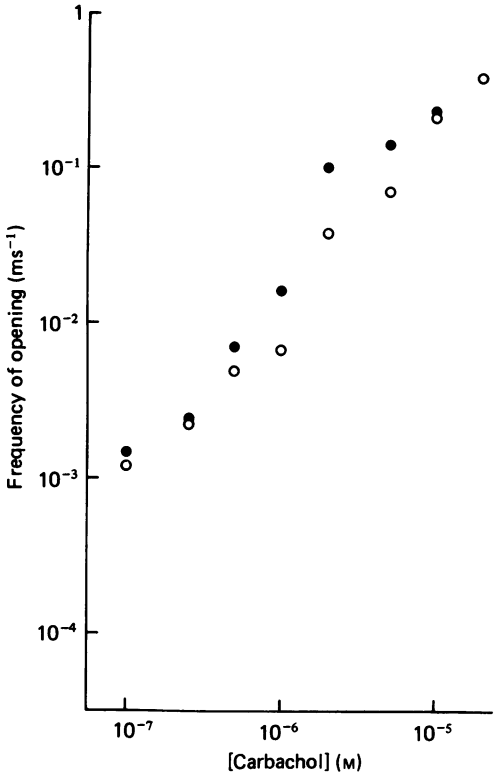

Fig. 3. A log–log plot of the slow decay constant of the closed-time distribution *versus* carbachol concentration. The slow decay constant is interpreted here as the frequency of channel opening. The mean is plotted as filled circles and the median is plotted as open circles. At 20 μM-carbachol the mean had no significance so it was not plotted (see text for explanation). Below 0·5 μM-carbachol frequencies are corrected for different electrode tip areas as described in the text.

This helped to extend the range of carbachol concentrations that could be studied.

The variation in channel opening frequency was studied further by analysing eighteen experiments with borosilicate electrodes (resistances between 10 and 20 MΩ) filled with 2 μM-carbachol. In these experiments, the frequency of opening varied from 0·0049 to 0·51 ms^{-1} (Fig. 4). There was no significant correlation, as judged by linear regression analysis, between opening frequency and electrode resistance in these experiments. It has previously been noted that electrode resistance and frequency of spontaneous openings are inversely correlated, when aluminasilicate electrodes are used (Jackson, 1984). The failure to detect a similar

correlation with borosilicate electrodes probably reflects differences in tip geometry. Aluminasilicate electrodes are steeply tapered at the tips so that variations in area are the primary source of variation in resistance. In the less steeply tapered borosilicate electrode tips, variations in length at the tip can also contribute to variations in electrode resistance (Sakmann & Neher, 1983). Because variations in resistance of aluminasilicate electrodes may give rise to noticeable variations in the number of channels in a patch (Jackson, 1984), aluminasilicate electrodes with a narrow range of resistances were used.

Variations in the density of receptors is the most important source of variation in the number of channels in a patch. The density of acetylcholine receptors in the membranes of cultured rat muscle fibres has been studied by quantitative autoradiography with radiolabelled α-bungarotoxin (Land *et al.* 1977). The density of toxin binding sites is highly variable, with occasional foci of high density. Occasional patches with extremely high channel activity were found in the present study. It has been shown that sites of high single-channel activity correlate well with foci of high toxin binding site density (Jackson, Christian & Lecar, 1979).

In Fig. 3 both the mean and the median of the frequency of opening are plotted. The mean is always higher than the median, consistent with a distribution that is skewed towards high values. This skewness results from the occasional high receptor density patches. The median is less sensitive than the mean to these outliers or anomalously high values, and thus should be a less variable measure of receptor density.

In two of nine data sets collected with 20 μM-carbachol, the channel activity was so high that there were few intervals during which all channels were closed. In these two data sets a kinetic analysis was impossible. Only after some desensitization had set in (discussed below) could a baseline current be clearly identified. A closed-time distribution could not be analysed in such data sets, and a mean value of the opening frequency at 20 μM-carbachol would have little meaning, because the two highest values could not be included. Hence, no mean is plotted at 20 μM in Fig. 3. The median can still be determined at 20 μM, and a value is plotted. Thus, the median can be used to obtain a useful value at high ligand concentration. Since the median also has the advantage of being less sensitive to outliers and high receptor density patches, the median of λ_2 will be used in subsequent analysis.

In summary, efforts have been made to account for, and to compensate for, variations in electrode tip area and channel density. This facilitates interpretation of λ_2, the slow decay constant of the closed-time distribution, as an apparent frequency of opening, which reflects channel activation kinetics. Evidence presented below indicates that when λ_2 is measured immediately after seal formation, it is only slightly affected by desensitization.

The kinetics of channel closing

The kinetics of channel closing was studied by analysing the distributions of isolated open times. Isolated open times are open times flanked on either side by times during which all channels are closed (Fig. 1*B*). Fitting eqn (3) to isolated open times, as described in Methods, indicated that in all but a few data sets a sum of two exponentials fitted open times much better than a single exponential. For nearly half

of the data sets, the addition of a third exponential produced a small, but statistically significant, improvement in the maximum likelihood.

A total of eighty-eight data sets could be divided into three groups based on the likelihood ratio of two and three exponential fits. When the logarithm of the likelihood ratio is less than three, the improvement in fit afforded by a third exponential is not significant at the 5% level. More than half the data fell into this group. When the likelihood ratio was between three and ten, the parameters derived from two exponential fits were consistent, and generally were similar to those of the first group (for a given concentration of carbachol). Re-examination of the results of closed-time fits of these two groups also revealed no differences. The parameters for the three exponential fits to open times were usually very poorly defined by a given data set and highly variable from one data set to the next. For these data the relatively weak violation of the two-exponential hypothesis did not appear to compromise the consistency of the parameters obtained from the two-exponential fit. The third group consisted of seven data sets in which the likelihood ratios were seventeen or more. The parameters obtained from two exponential fits to these data sets were noticeably different from those obtained from the other data sets collected at the same concentration of carbachol. These seven data sets were excluded from further analysis.

It should be noted that four of the seven sets of open times of the latter group, which were so well fitted by a sum of three exponentials, were collected on one day and two more from this group were collected on another day. This suggests that the variability is the result of some as yet undetermined aspect of the tissue culture conditions that occurs infrequently to promote production of different populations of receptor. Experiments done with cultures that were 11 rather than 7 days old were best fitted by two exponentials, with identical parameters. Thus, variation in developmental progress is not likely to be a critical factor. Low and variable levels of another receptor population are a likely cause of the frequent slight violations of the hypothesis that two exponentials is the better model.

Testing the stochastic theory of a many channel system

The eighteen data sets collected with 2 μM-carbachol provide an opportunity to test how effectively the mathematical theory of stochastic behaviour of a many channel system, as embodied in eqn (3), can be used to analyse open times, since these data sets vary dramatically in their level of channel activity, and hence in the extent to which eqn (3) corrects for multiple openings. Figure 4 shows that parameters of the open-time distribution (the fast and slow decay constants and the fraction of brief openings) did not vary in a consistent manner with the frequency of channel opening. No statistically significant correlation was found. A similar analysis of fifteen experiments with 5 μM-carbachol gave a similar result (data not shown). These results are consistent with the hypotheses that eqn (3) corrects effectively for different levels of channel activity and that channel closing kinetics are not correlated with receptor density.

A comparison of open-time parameters obtained from experiments with alumina-silicate and borosilicate electrodes also supports the effectiveness of eqn (3). As mentioned above, with 0·5 μM-carbachol eight experiments were carried out with

aluminasilicate electrodes and six with borosilicate electrodes. Because there is approximately four times as much channel activity in patches formed by the larger tipped aluminasilicate electrodes, the effect of multiple openings is higher in these data. When a comparison was made, no differences were found in any of the open-time parameters obtained using electrodes fabricated from the different glasses.

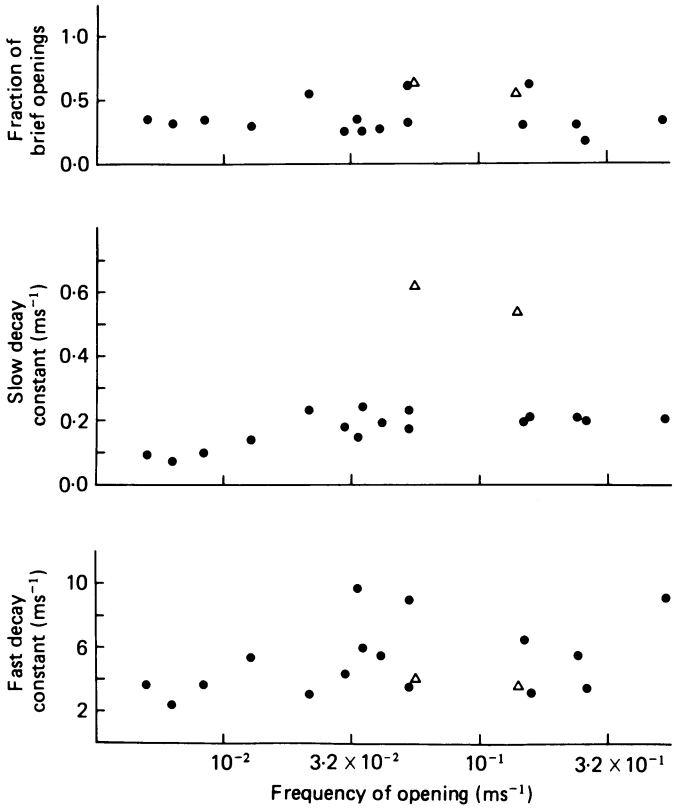

Fig. 4. Open times from eighteen data sets collected at 2 μM-carbachol were fitted by eqn (3) with a single-channel open-time density having two exponential components. The parameters are plotted *versus* the logarithm of the frequency of opening. This plot illustrates the lack of correlation between the kinetic parameters of channel closure and the level of channel activity in a patch. Two particular sets of open times were much better fitted by a sum of three exponentials. The parameters obtained from these data sets are indicated with triangles instead of circles.

Open-time distributions from two records obtained with 2 μM-carbachol, but with very different levels of channel activity, are compared in Fig. 5. The distribution of isolated channel open times is strongly attenuated in a patch of membrane exhibiting a high level of channel activity (Fig. 5*B*). If one were to fit these open times to a sum of two exponentials, the resultant parameters would be quite far from the actual parameters relevant to the process of channel closure. This point has already been made with simulated data (Jackson, 1985). The single-channel open-time distributions, calculated from parameters obtained by fitting eqn (3), are drawn in both

panels of Fig. 5. In Fig 5*B*, taken from a data set with high channel activity, the computed distribution is quite different from the observed distribution of isolated openings. These two distributions are very similar for a patch exhibiting low channel activity (Fig. 5*A*). Clearly, the distribution of isolated channel open times is strongly influenced by the channel activity in the patch, but the parameters determined by this analysis are not.

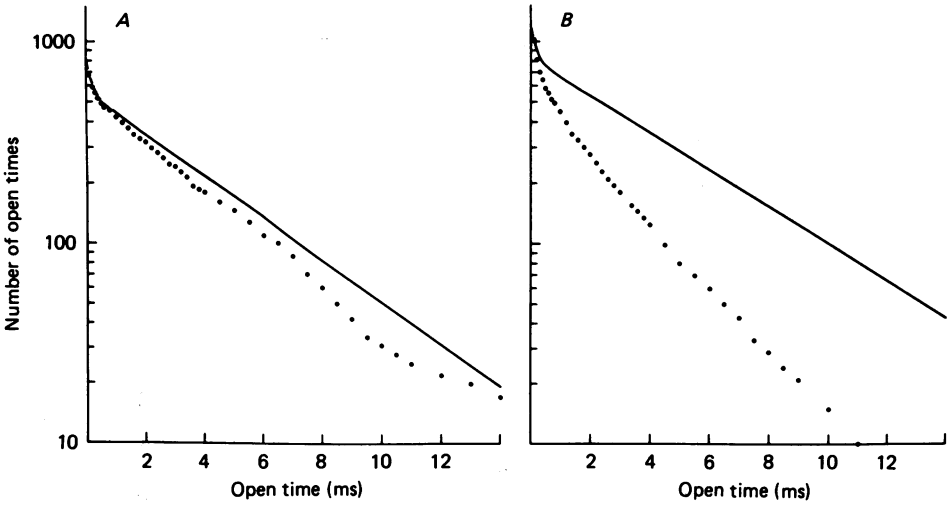

Fig. 5. A comparison of the cumulative distribution of isolated openings (dots) with the cumulative open-time distribution of a single channel (smooth curve), computed from parameters obtained by fitting eqn (3) to isolated openings. In *A* the level of channel activity was low as in Fig. 1*A*. In *B* the level of channel activity was high as in Fig. 1*B*. Both data sets were obtained with thick-walled borosilicate electrodes filled with 2 μM-carbachol. The isolated openings have a very different distribution in *A* and *B*, but the computed single-channel open-time distributions, determined from each data set independently, as described in Methods, are quite similar. The parameters for the best fits in *A* were $\nu_1 = 6{\cdot}0$ ms^{-1}, $\nu_2 = 0{\cdot}24$ ms^{-1}, $y_1 = 0{\cdot}26$, for open times and with $\lambda_2 = 0{\cdot}029$ ms^{-1} determined separately from closed times (only one exponential). In *B* the parameters were $\nu_1 = 5{\cdot}5$ ms^{-1}, $\nu_2 = 0{\cdot}21$ ms^{-1}, and $y_1 = 0{\cdot}30$, for open times, and with $\lambda_1 = 32$ ms^{-1}, $\lambda_2 = 0{\cdot}21$, $x_1 = 0{\cdot}26$ determined separately from closed times.

The concentration dependence of channel closing

Parameters from fits of a sum of two exponentials to open times were averaged together for each concentration and are plotted in Fig. 6. Only y_1, the fraction of brief openings, shows a clear variation with agonist concentration. Larger standard deviations are evident at high carbachol concentrations, reflecting the larger corrections required at higher channel activities. This trend is especially strong for ν_1, the rapid decay constant. Linear regression shows that there is no significant correlation between the two decay constants, ν_1 and ν_2, and the carbachol concentration. Thus, these data do not indicate blockade of open channels by agonist (Sine & Steinbach, 1984*b*; Colquhoun & Sakmann, 1985).

Above 1 μM-carbachol, long-duration openings predominate and below 1 μM-carbachol short-duration openings predominate. Furthermore, as discussed above, the fraction of brief closures also changes significantly at 1 μM-carbachol (Fig. 2*A*).

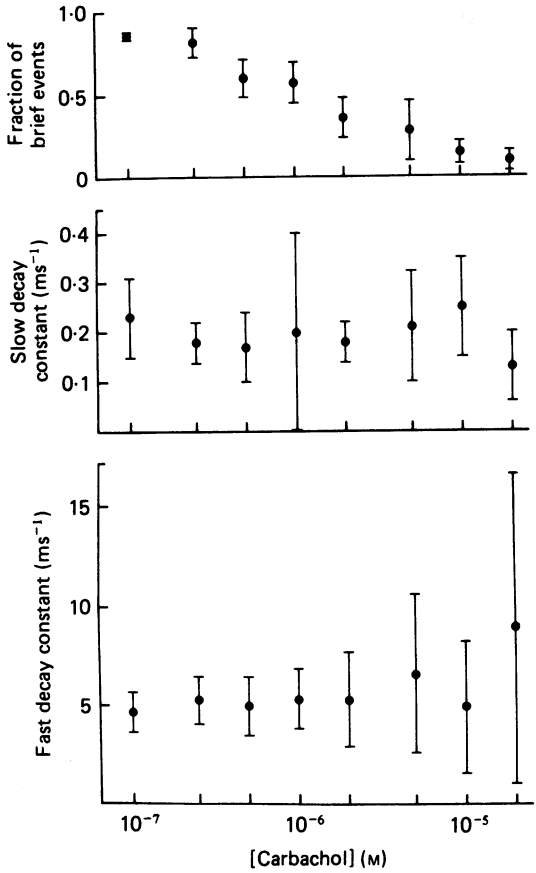

Fig. 6. The fast decay constant ν_1, the slow decay constant ν_2, and the fraction of brief openings y_1, for the single-channel open-time distribution, were averaged for each concentration of carbachol and plotted *versus* the logarithm of the carbachol concentration. Standard deviations rather than standard errors are indicated for each point to illustrate the increase in error at higher agonist concentrations. From five to sixteen data sets (with an average of eleven) were used for each concentration.

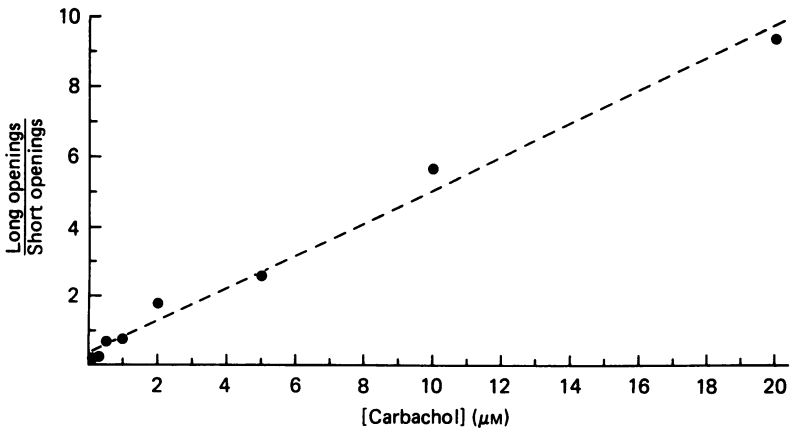

Fig. 7. The fraction of long-duration openings is divided by the fraction of short-duration openings (y_2/y_1) and plotted *versus* the carbachol concentration. The best-fitting line is also displayed.

This suggests that the 22 ms^{-1} component of closed times described above is associated with long-duration channel openings.

In a two-binding-site model of receptor activation, where singly liganded receptor channels close more rapidly than doubly liganded receptor channels, the ratio of long openings to short openings should increase linearly with agonist concentration (Sine

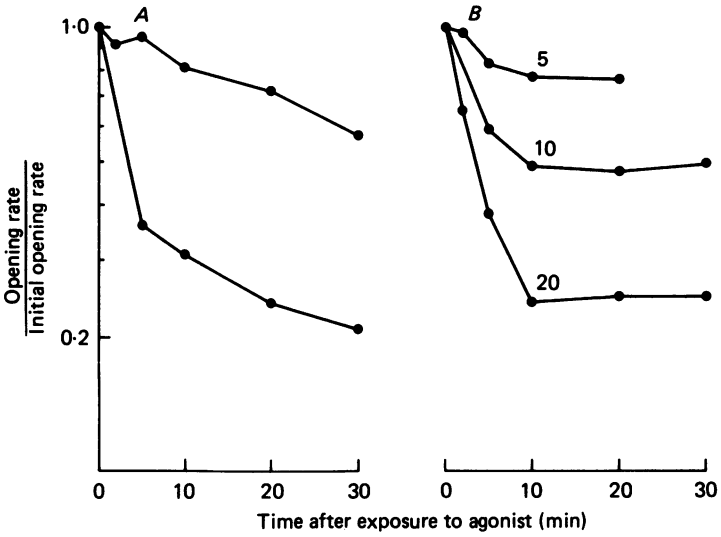

Fig. 8. The logarithm of the frequency of opening is plotted *versus* time after seal formation. All data are normalized to an initial value of one. The decline in opening frequency illustrates receptor desensitization. In *A*, two separate experiments with 10 μM-carbachol are plotted to illustrate the typical result in which a rapid phase of desensitization is evident, and an atypical result in which a rapid phase is not evident. In *B*, the data for each concentration of carbachol are averaged together and plotted. The concentrations were 5 μM (four experiments), 10 μM (six experiments), and 20 μM (six experiments), as indicated above each trace.

& Steinbach, 1984*a*; Colquhoun & Sakmann, 1985). This ratio (y_2/y_1 in terms of the parameters of eqn (4)) is plotted in Fig. 7, and shows an essentially linear dependence on the agonist concentration. It should be noted that the zero concentration intercept is 0·37 rather than zero. This is higher than the lowest values of y_2/y_1 seen with 0·10 and 0·25 μM-carbachol. Although the small positive intercept is not statistically significant, slowly closing channels have been observed in unliganded (Jackson, 1986*a*, *b*) and singly liganded (Chabala, Gurney & Lester, 1986) receptor channel openings. A small positive intercept in Fig. 7 is consistent with the notion that open times of singly liganded receptor channels are described by two exponentials. There is no evidence for a high concentration asymptote in Fig. 7 as has been observed in frog end-plate by Colquhoun & Sakmann (1985) with the agonist suberyldicholine.

Changes in channel kinetics during desensitization

With carbachol concentrations of 5 μM or higher there is a reduction in channel activity over time. This desensitization has been investigated by collecting data at

various times after seal formation. Care was taken to maintain gentle positive pressure inside the patch electrode until immediately before seal formation, avoiding agonist dilution within the electrode tip. The time to approach the cell and form the seal was usually short (approximately 10 s) and data sampling could be initiated within a few seconds of seal formation. When an electrode took an excessively long

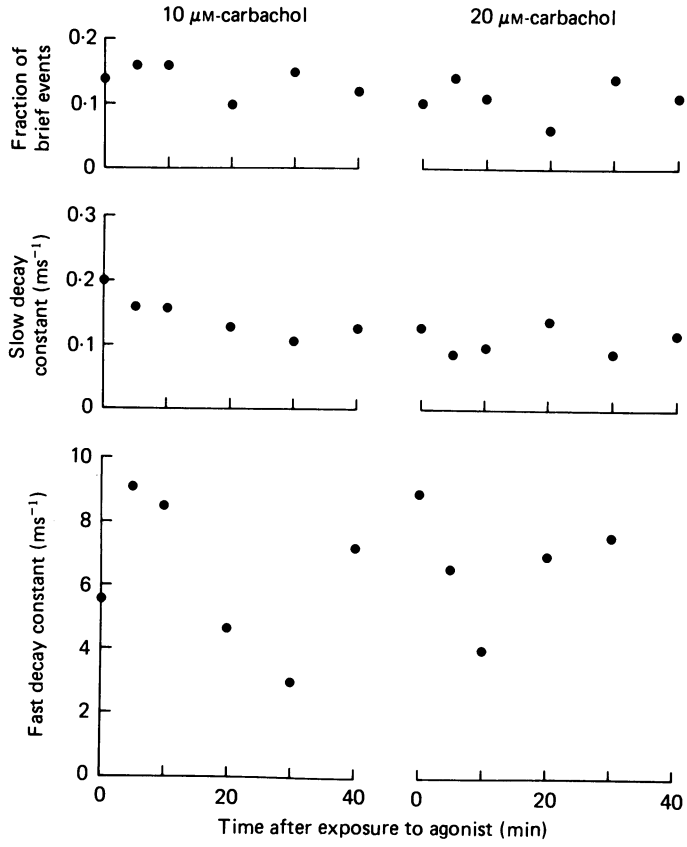

Fig. 9. The fast decay constant ν_1, the slow decay constant ν_2, and the fraction of brief openings y_1, of single-channel open-time distributions were averaged for each time after seal formation and plotted. These parameters show no significant changes over a time period during which examination of Fig. 8 shows that significant desensitization occurs. The standard errors are comparable to the scatter in the data, and the standard deviations were similar to those displayed at the same concentrations in Fig. 6. Five separate experiments were used to generate the plots for 10 μM-carbachol and six experiments were used to generate the plots for 20 μM-carbachol.

time to seal, the experiment was thrown out. Given the small tip sizes of the thick-walled glass electrodes used at high concentrations, it is unlikely that carbachol leakage from the electrode produced appreciable desensitization in the few seconds before electrode contact. Thus, the total dead time in these experiments of the kinetics of desensitization was estimated to be 15 s. Data were then collected for a period of approximately 20–30 s.

The frequency of opening was determined from data sets collected at various times

after seal formation. Two examples are shown in Fig. 8*A*. Two components of desensitization are often evident, as has been reported previously (Feltz & Trautmann, 1982). The faster component of desensitization is occasionally absent (Fig. 8*A*). Averages from several patches are shown in Fig. 8*B*. There is a clear increase in the rates of desensitization with concentration of carbachol, with only a 25% decrease in channel activity occurring with 5 μM-carbachol, and a 76% decrease at 20 μM-carbachol. In addition, the first record collected immediately after seal formation was examined to monitor decline in channel activity. Decreases during this initial recording period (20–30 s) were small, and are consistent with the decreases between the first few recording periods indicated in Fig. 8.

Open times were analysed at various times after seal formation. The parameters are plotted in Fig. 9. Although they are variable (as has been noted already for the high-concentration data presented in Fig. 6), no trend is evident. Thus, within the error of these experiments, the kinetics of channel closure do not change as desensitization progresses.

DISCUSSION

Analysis of multi-channel patches

The extension of the Colquhoun & Hawkes (1981) theoretical formalism of single-channel gating kinetics to a system with an arbitrary number of channels (Jackson, 1985) has been used to analyse channel kinetics in membrane patches with intermediate levels of channel activity. The methods of analysis presented here have been used to study the variation in acetylcholine receptor channel gating kinetics with concentration of the agonist carbachol. In addition, channel gating kinetics have been studied over a period of time during which a significant amount of receptor desensitization occurs.

Standard deviations shown in Fig. 6 illustrate the progressively larger errors at higher concentrations of carbachol. These larger errors are due to larger corrections implemented by this method when higher levels of channel activity are induced by higher concentrations. At 20 μM-carbachol some data sets have very few time intervals during which all channels are closed. At higher concentrations of carbachol, most data sets would contain no such intervals and, without desensitization, it would be impossible to analyse these data with the methods employed here.

For a given concentration of carbachol the kinetic parameters associated with channel closing did not vary with receptor density (Fig. 4). A similar observation has been made by Schuetze, Frank & Fischbach (1978) using noise analysis. Previous studies of acetylcholine sensitivity as a function of receptor density are consistent with this observation (Land *et al.* 1977).

Interpretation of brief closures

An increase in the fraction of brief closures was observed near 1 μM-carbachol. A similar increase has been interpreted as evidence for open-channel block (Colquhoun & Sakmann, 1985). The correlation between the increase in brief closures, and the increase in long openings (compare Fig. 2*A* and Fig. 6), suggests that the brief closures are not due to open-channel block, but are associated with long-duration

openings, and presumably with doubly liganded receptors. Correlation analysis has also shown that with the agonist suberyldicholine most brief closures are associated with long openings (Colquhoun & Sakmann, 1985; Jackson, 1986*b*; Sine & Steinbach, 1986). Brief openings are more often seen as isolated events (Sine & Steinbach, 1984*a*; Colquhoun & Sakmann, 1985; Sine & Steinbach, 1986; Auerbach & Lingle, 1986).

The decay constants of channel closure do not exhibit a detectable increase with concentration of carbachol (Fig. 6). This also argues against an appreciable rate of carbachol blockade of open channels. At 10 kHz, parameters associated with the fast component of closures were similar to those seen at 5 kHz. If agonist blockade is occuring in these experiments, the events must be very brief. The absence of blockade by carbachol at low and intermediate carbachol concentrations is consistent with the results of Sine & Steinbach (1984*b*), who suggested that carbachol is a relatively poor channel blocker as compared with other agonists. Higher concentrations of carbachol are necessary to produce a detectable blocking effect.

Variation in closing kinetics with carbachol concentration

The variation in the proportion of rapidly and slowly closing channels with carbachol concentration confirms some other studies (Takeda & Trautmann, 1984; Colquhoun & Sakmann, 1985; Labarca *et al.* 1985) and supports the interpretation that the brief openings are openings of singly liganded receptor channels. The linear increase in the ratio of long- to short-duration openings, shown in Fig. 7, has been observed only at low concentrations of the agonist suberyldicholine at frog muscle end-plates (Colquhoun & Sakmann, 1985). Sine & Steinbach (1984*a*) did not observe a large change in the relative weights of long- and short-duration openings with agonist concentration for nicotinic receptors in a clonal cell line. It is difficult at this stage to determine the origin of these discrepancies.

Multiple open states

The remnant of a slow component of openings at low carbachol concentrations (Fig. 7) suggests that a singly liganded receptor can have two open states. This has also been suggested by Chabala *et al.* (1986). An unliganded receptor may also have two open states (Jackson, 1986*a*, *b*). The persistence of brief openings at high concentrations suggests that a doubly liganded receptor can have two open states (Sine & Steinbach, 1984; Colquhoun & Sakmann, 1985; Auerbach & Lingle, 1986). Whether these observations are the result of different receptor populations or multiple open states of each state of receptor occupancy remains to be seen. Nevertheless, the open-time distributions at extreme concentrations are dominated by single components, with a clear correlation between open-state lifetime and concentration.

The present study suggests that with carbachol there is a single open state of a doubly liganded receptor channel, so there is no ambiguity of assignment. With unliganded and singly liganded receptors, there is an ambiguity. Openings from these two states are almost entirely accounted for by the brief-duration component, so the weight of the brief component in an open-time distribution is a reasonable measure of the number of singly liganded receptor channel openings.

Desensitization

Desensitization is manifest as a reduction in channel activity over a time period of the order of minutes. The analysis provided here suggests that the only kinetic parameter that changes appreciably in this time period is the frequency of opening. There are no dramatic changes in the kinetics of channel closure over this time period. At this level of analysis desensitization is effectively a removal of channels from the patch.

Data of the form in Fig. 8 may be useful in the study of the kinetics of desensitization and its dependence on ligand concentration. The time course of desensitization at 10 μM-carbachol is comparable to that seen by Feltz & Trautmann (1982) (a single exponential with a time constant of 135 ms) with the same concentration of carbachol at the frog end-plate. Desensitization is considerably faster at higher concentrations, with a time course approaching 1 s. However, there is a supralinear concentration dependence (Feltz & Trautmann, 1982), and the concentrations used here are much lower than those which produce desensitization on a time scale of a few seconds.

In the present study, no more rapid desensitization processes were resolved early in the recordings, 15 s after initial exposure to agonist. This statement is based on examination of changes in mean current in records collected fom 15 to 35 or 45 s after seal formation. With these experiments it would not be possible to observe a desensitization process that is completed within 15 s. Reducing the concentration of carbachol did not bring out a fast component. A large undetected component of desensitization would produce saturation and even downward curvature in the plot of opening frequency *versus* concentration. Figure 3 shows slight saturation, but the analysis described below indicates that there is more saturation in short-duration openings.

While the time course of desensitization is consistent with that reported by Feltz & Trautmann (1982) at 10 μM-carbachol, the extent of desensitization is not as great, and was variable from patch to patch (Fig. 8*A*). This variability may be a function of receptor phosphorylation (Huganir, Delcour, Greengard & Hess, 1986; Middleton, Jaramillo & Schuetze, 1986). Preliminary experiments with forskolin and phorbol esters produce large changes in the extent of desensitization (data not shown).

The two-binding-site model

The variation in the proportion of rapidly and slowly closing channels with concentration (Figs 6 and 7) is a compelling reason to use the two-binding-site model as a basis for further analysis. This model has already received much attention (Katz & Thesleff, 1957; Adams, 1975; Dionne *et al.* 1978; Karlin, 1980; Sine & Taylor, 1981; Colquhoun & Hawkes, 1982; Colquhoun & Sakmann, 1985; Jackson, 1986*a*, *b*).

$$\begin{array}{ccccc} & & K_1 & & K_2 & & \\ & \mathrm{C} & \rightleftharpoons & \mathrm{CA_1} & \rightleftharpoons & \mathrm{CA_2} & \\ \alpha_0 & \uparrow\downarrow & \beta_0 & \alpha_1 & \uparrow\downarrow\ \beta_1 & \alpha_2 & \uparrow\downarrow\ \beta_2 \\ & \mathrm{O} & \rightleftharpoons & \mathrm{OA_1} & \rightleftharpoons & \mathrm{OA_2} & \\ & & J_1 & & J_2 & & \end{array} \qquad (5)$$

In this scheme C represents a closed channel, O represents an open channel, and A represents a molecule of bound ligand. Lower-case Greek letters in the scheme denote rate constants, and upper-case italic roman letters denote dissociation constants. Lower-case roman letters will be used below to denote ligand association and dissociation rates.

Opening kinetics of the two-binding-site model

The two-agonist model has three distinct closed states, and thus a one-channel system would have a closed-time distribution consisting of three exponential terms (Colquhoun & Hawkes, 1982). However, if two of the decay constants are very slow, and there are many channels, the frequent activation of other channels would mask the slower processes of the one-channel system. This can be seen by noting that the closed-time distribution of the N-channel system can be approximated as

$$p_{Nc} \simeq [w_1 e^{-\omega_1 t} + w_2 e^{-\omega_2 t} + w_3 e^{-\omega_3 t}] e^{-(N-1)\omega_3 t}, \quad (6)$$

where the last exponential factor represents activations of the $N-1$ channels other than the most recent one to close. This follows from eqn (21) of Jackson (1985). $(N-1)\omega_3$ in this expression is essentially the frequency of opening. It is a sum of a quantity f_1, the frequency of a receptor being activated by a single ligand, and a quantity f_2, the frequency of a receptor being activated by two ligands. If $(N-1)\omega_3$ is much larger than ω_2, but smaller than ω_1, then eqn (6) reduces approximately to a sum of two exponentials, the slow decay constant of which is approximately $(N-1)\omega_3 \simeq N\omega_3$, and the fast decay constant of which is approximately ω_1.

With only two exponential components evident in closed-time distributions, we can assign these components to specific kinetic processes as follows. Short closures have already been shown to be associated with long openings, and thus presumably with doubly liganded receptors. We can therefore identify short closures with the transition from CA_2 to OA_2. Long-duration closed times, with decay constant λ_2, are assigned to the combined processes of ligand binding to receptor and subsequent channel opening.

The rapid decay constant of the closed-time distribution, λ_1, is therefore approximately equal to $\beta_2 + 2k_{-2}$, where β_2 is the rate of channel opening from CA_2 and k_{-2} is the rate of ligand dissociation from a doubly liganded receptor. The fraction of brief closures, x_1, is then $\beta_2/(\beta_2 + 2k_{-2})$. These relationships were originally based on a theoretical analysis of a one-channel system, but the fact that λ_1 and x_1 obtained from analysis of closed times are roughly constant from 1 to 10 μM-carbachol (Fig. 2*B*) suggests that this analysis of Colquhoun & Sakmann (1981) can be used here. This gives $\beta_2 = 2{\cdot}8$ ms^{-1} and $2k_{-2} = 17$ ms^{-1}.

An approximate expression for the frequency of opening can be obtained by calculating the fraction of closed channels in a particular state of receptor occupancy at equilibrium, and multiplying by $N\beta_1$ or $N\beta_2$. Openings from each state of occupancy then occur with the following frequencies:

$$f_1 = N\beta_1[A]/K_1(1 + [A]/K_1 + [A]^2/K_1K_2), \quad (7)$$

and

$$f_2 = N\beta_2[A]^2/K_1K_2(1 + [A]/K_1 + [A]^2/K_1K_2). \quad (8)$$

The observed frequency is then f_1+f_2. Equations (7) and (8) are approximate since they include times spent in CA_1 and CA_2 immediately after closing, while these equations are intended to represent new activations of receptors rather than repeated activations. This is a reasonable approximation since the rate of ligand dissociation is approximately six times greater than the rate of channel opening for CA_2. Thus, a relatively small fraction of time spent in closed states of the doubly liganded receptor occurs after an opening. Reopenings from CA_1 should be well separated, as subsequent analysis indicates that β_1 is very low. Thus, they would contribute almost entirely to the longer closed times.

Assignment of various rate processes in closed-time distributions is somewhat controversial (Colquhoun & Sakmann, 1985; Sine & Steinbach, 1986). This is partly because additional exponential components are often observed. In the present study no such components are evident, and so assignment of the components of the closed-time distribution to specific rate processes is less ambiguous. Any additional component of closures would have to be either quite slow, or so small that it is difficult to see how it could make a significant contribution to the activation process.

Response versus agonist concentration

The theoretical expressions for the frequency of channel opening of singly and doubly liganded receptors (eqns (7) and (8)), can now be compared with appropriate quantities derived from the experimental data. The experimentally determined fractions of long- and short-duration openings, y_1 and y_2, are multiplied by the median frequencies of opening (i.e. λ_2, the slow decay constant of the closed-time distribution, plotted in Fig. 3) to obtain $y_1\lambda_2 = f_1$ and $y_2\lambda_2 = f_2$. Figure 10 displays a log–log plot of f_1 and f_2 *versus* the concentration of carbachol.

As discussed above, even at the highest concentrations used, desensitization did not present a problem in measuring the opening frequency. Examination of the time course of desensitization (Fig. 8) suggests that at 20 μM-carbachol desensitization lowers the frequency of opening by about 9% before the first measurement (assuming a 15 s dead time as discussed above and including half of the typical sampling period to estimate the time that elapses). The median frequencies of opening were accordingly corrected at both 10 and 20 μM-carbachol. No corrections were necessary at lower concentrations. Estimates of parameters presented below were insensitive to the precise value of this correction.

It is evident in Fig. 10 that at low agonist concentrations brief openings, f_1, increase as the first power of the agonist concentration and long openings, f_2, increase as the second power of the agonist concentration. This result lends considerable weight to the two-binding-site model, and to the interpretation of slowly closing and rapidly closing channels in terms of the respective gating of doubly and singly liganded receptors.

Response concentration plots and models of receptor activation

The plots in Fig. 10 were compared with predictions of various models by curve-fitting techniques. The 0·1 μM data are not included in these fits, because at such a low concentration of carbachol spontaneous openings accounted for a significant fraction

of events. Fitting was carried out by minimization of the logarithms of the sums of square differences between an appropriate theoretical expression and the data points in Fig. 10. The computer program used to carry out this minimization was the same as that used to maximize the likelihood (See Methods). To evaluate the quality of the fits, the variance of the data was estimated as the standard error of the mean at each

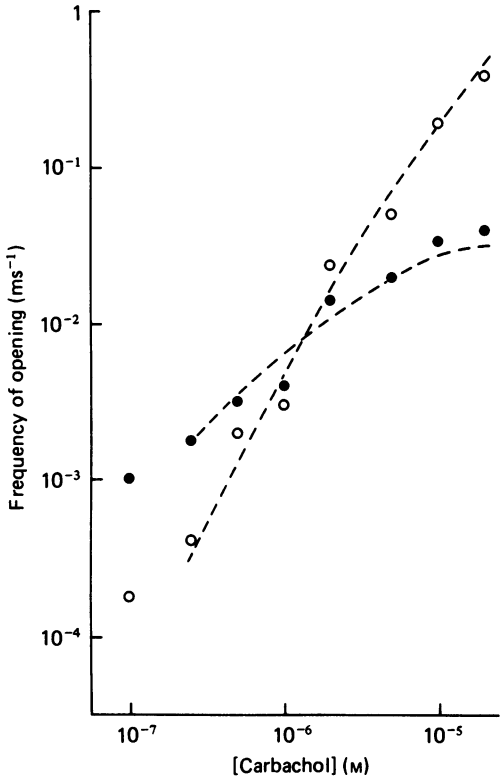

Fig. 10. The median frequency of opening from Fig. 3 was decomposed into a frequency of opening of rapidly closing channels (●) and a frequency of opening of slowly closing channels (○) as described in the text. The logarithms of these frequencies are plotted *versus* the logarithms of the carbachol concentration. The drawn dashed curves are examples of good, but not unique (see text), fits of eqns (7) and (8). The parameters chosen for this plot are $N\beta_2 = 300\ ms^{-1}$, $K_1K_2 = 5{\cdot}5 \times 10^4\ \mu M^2$, $N\beta_1 = 0{\cdot}040\ ms^{-1}$, $K_1 = 5{\cdot}1\ \mu M$. The points at 0·1 μM-carbachol were not used to generate the fit because of a significant contribution from spontaneous openings.

concentration, although the standard error is not an ideal estimate of the variance of the median and the distribution of receptor densities is skewed as noted in Results. The standard errors of the mean were on average 25% per point. A χ^2 for the goodness-of-fit was estimated as the ratio of the sum of squares error to the estimated variance.

When simple linear or quadratic expressions were fitted to each plot of Fig. 10 separately, the χ^2 test rejected these models with very high confidence ($P < 0{\cdot}01$). Simple Hill equations, with Hill coefficients of one and two for the filled and open circles respectively, improved the fits considerably. The fit was especially good for

the filled circles (openings produced by binding one ligand), giving a χ^2 of 0·75 ($P > 0·5$, for four degrees of freedom) and an apparent K_1 of 9 ± 8 μM. For the open circles (openings produced by binding two ligands), the fit was marginal, with an apparent K_2 of 70 ± 60 μM ($P < 0·10$). Estimating χ^2 as the ratio of the sum of squares for the saturating and non-saturating models also indicates statistically significant saturation in both plots.

The pair of equation (eqns (7) and (8)) was then fitted simultaneously to the data of Fig. 10. Although the model contains many undetermined quantities, the equations contain only four independent parameters. They are K_1, $N\beta_1$, K_1K_2, and $N\beta_2$.

Evaluation of an equivalent binding site model

One can test the hypothesis of equivalent binding sites by setting K_1 and K_2 as equal. When this is done a marginal fit was obtained with $K = 13 \pm 7$ μM, $N\beta_1 = 0·09 \pm 0·03$, and $N\beta_2 = 0·9 \pm 0·7$ ms^{-1} ($P < 0·1$).

While one cannot reject this model on the basis of goodness-of-fit, a compelling argument for a non-equivalent binding site model can be made if we consider some of the consequences of assuming equivalent binding sites. At frog end-plates, carbachol dose–response curves reach half-maximal saturation at 200–300 μM (Dionne *et al.* 1978). If the binding sites are equivalent, then the analysis here suggests that half-saturation would occur at 13 ± 7 μM. In addition, N, the numer of channels in the patch, can be estimated from the parameter $N\beta_2$, determined in the curve fit, if we take β_2 (the rate of opening with a doubly liganded receptor) as 2·8 ms^{-1} from above analysis of closed-time distributions. This yields $N = 0·3 \pm 0·2$, which is clearly impossible. Aside from the much higher density of α-bungarotoxin binding sites (Land *et al.* 1977), any patch with high concentrations of agonist routinely shows multiple openings of more than ten channels (data not shown). Thus, two independent consequences of an equivalent binding site hypothesis are not tenable.

Fitting to a non-equivalent binding site model

Allowing the two dissociation constants, K_1 and K_2, to vary independently improved the quality of the fit by a small amount ($P < 0·2$) over that of the equivalent binding site model, and yielded very different values for K_1 and K_2. The information matrix that resulted from these fits could not be inverted, indicating that the four parameters are not uniquely defined by the data. The sum of squares surface in parameter space was explored by carrying out large numbers of fits with many different initial guesses of the parameters. This exploration indicated that the reason the parameters are not uniquely defined is that $N\beta_2$ and K_1K_2 are highly correlated. The ratio of these two quantities was highly conserved and was always 0·0056 μM^{-2} ms^{-1}. The parameter $N\beta_2$ was never below 100 ms^{-1}, and K_1K_2 was never below $1·8 \times 10^4$ μM^2 even when much lower initial guesses were made. Fits that converged with higher values of $N\beta_2$ had a slightly lower sum of squares, but any set of parameters obtained with $N\beta_2$ above 100 ms^{-1} should be considered as equally consistent with the data. For all of the fits, $N\beta_1$ was 0·040 ms^{-1} and K_1 was 5·1 μM.

Thus, the data define these two parameters, but only define the ratio of $N\beta_2$ and K_1K_2, and their lower limits. The smooth curves drawn through the data in Fig. 10 are an example of the equations plotted for particular choices of the parameters

(see legends of Fig. 10). The high correlation between $N\beta_2$ and K_1K_2 suggests that the quadratic terms in the denominators of eqns (7) and (8) are negligible. To test this a final curve fit was carried out, leaving out these terms. The quantity $N\beta_2/K_1K_2$ could thus be treated as a single parameter, reducing the number of parameters to three. This fit converged and fitted the data precisely as well as the more general model, yielding the following parameter values: $K_1 = 5 \pm 3$ μM; $N\beta_1 = 0{\cdot}04 \pm 0{\cdot}02$ ms^{-1}; $N\beta_2/K_1K_2 = 0{\cdot}0056 \pm 0{\cdot}0021$ ms^{-1} μM^{-2}.

Non-equivalent binding sites and estimates of parameters

The rejection of the equivalent binding site model for reasons stated above justifies further analysis in terms of a model with non-equivalent binding sites. The question is then either what is the binding affinity of the second binding site? or what is the number of channels in a patch? Unfortunately, the data presented here do not allow a determination of these two parameters individually, but only of their product. Limits to these quantities can be inferred, however. With 100 as the lower limit for $N\beta_2$, estimated above in curve fits to eqns (7) and (8), we can at least estimate a lower limit of 36 to the median number of active acetylcholine receptor channels in a patch of membrane sealed off by a patch electrode fabricated from thick-walled borosilicate glass. This gives a lower limit of 3·5 mM for K_2.

The lower limit of thirty-six channels per patch is well below an estimate based on autoradiographic determination of toxin binding site density (Land *et al.* 1977; Jackson, 1986*a*). Correcting to the area of membrane patches in this study gives a value of 1200 receptors per patch. However, it is not sound to use such an estimate, because many studies suggest that there is a large discrepancy between the ligand-induced ion flux and the density of toxin binding sites (Caterall, 1975; Dionne *et al.* 1978), possibly because a large fraction of toxin binding sites are receptors that are silent, inaccessible, or inactive.

With so many other parameters known, one might hope to make a reasonable estimate of K_2 by comparing the dose–response curve predicted by the non-equivalent binding site model with experiments. It is readily shown that when the two binding constants are very different, the response is half-saturated at an agonist concentration of approximately K_2. The experimental values for half-saturation of carbachol responses in other preparations are very variable and model dependent; they are never above 800 μM, and are usually less (Dionne *et al.* 1978; Karlin, 1980). It is possible that half-saturating agonist concentrations for macroscopic responses are underestimated, since equivalent site models are assumed and an inhibitory binding site (Shiono, Takeyasu, Udgaonkar, Delcour, Fujita & Hess, 1984) is neglected. Alternatively, in order to reconcile the data of Fig. 10 with a value of K_2 significantly lower than 3·5 mM, it would be necessary to alter model (5), possibly by adding a second doubly liganded closed state. Such a state would then have to have a long lifetime to be missed in the closed-time distributions of Fig. 2.

As long as N is above its minimum of 36, one obtains values for K_2 that are dramatically larger than K_1 (5 μM, estimated above). For the minimum value of N, K_2 is 700 times larger than K_1; the ratio K_2/K_1 increases with N. Thus, these data are not at all consistent with the hypothesis that the two acetylcholine binding sites are equivalent and non-interacting. Either the binding sites have very different

affinities for carbachol or there is strong negative co-operativity of binding. Differences between the two binding sites have previously been revealed by biochemical (Karlin, 1980) and pharmacological (Sine & Taylor, 1981) probes. The difference in binding affinities to an agonist is a new observation that was not evident in previously reported macroscopic dose–response curves (Adams, 1975; Dionne *et al.*

TABLE 1. Values of rate constants

Opening rates
$\beta_0 = 2{\cdot}8 \times 10^{-5}$ ms^{-1}
$\beta_1 = 1{\cdot}1 \times 10^{-3}$ ms^{-1}
$\beta_2 = 2{\cdot}8$ ms^{-1}

Closing rates
$\alpha_0 = 5$ ms^{-1}
$\alpha_1 = 0{\cdot}9$ ms^{-1}
$\alpha_2 = 0{\cdot}2$ ms^{-1}

Binding rates
$k_2 = 5 \times 10^6$ s^{-1} M^{-1}
$2k_{-2} = 17$ ms^{-1}

Open–closed equilibrium constants
$R_0 = 5{\cdot}0 \times 10^{-6}$
$R_1 = 1{\cdot}2 \times 10^{-3}$
$R_2 = 14$

Closed-channel dissociation constants
$K_1 = 5$ μM
$K_2 = 3{\cdot}5$ mM

Open-channel dissociation constants
$J_1 = 0{\cdot}025$ μM
$J_2 = 0{\cdot}11$ μM

k_2, β_0, β_1, R_0, and R_1 are upper bounds. K_2 and J_2 are lower bounds. Although these quantities are bounds, they have a similar dependence on N so their ratios are well defined.

1978) because the macroscopic dose–response curve is dominated by long-duration openings at all but the lowest concentrations.

The analysis will now be completed with 36, the lower bound estimated above, chosen as the median number of channels in a patch. While this is somewhat arbitrary, the major points of the ensuing discussion are the disparities between the binding sites, which only become greater with increasing N.

These data, and data from a previous study from this laboratory (Jackson, 1986*a*), then make it possible to estimate every equilibrium constant in the two-binding-site model (Table 1). It should be noted that it was necessary to recompute the rate of spontaneous opening from already published data (Jackson, 1986*a*) by taking the median instead of the mean. This reduced the estimate of the spontaneous channel opening rate, β_0, by a factor of two. The previous estimate of β_0 was also multiplied by thirty-four because the lower bound of the number of active channels in a patch is used. We then obtain the values for opening rate constants shown in Table 1. The closing rates, also shown in Table 1, were taken as the appropriate decay constants from open-time distributions, or in the case of singly liganded and spontaneous openings, were taken as the reciprocal of the mean open time. The equilibrium constants for the open–closed transition were then computed by multiplying the opening rate by the mean open time. Finally, application of detailed balance provides estimates of dissociation constants for receptor with an open channel.

With the dissociation constant $K_2 = 3{\cdot}5$ mM and the dissociation rate $2k_{-2} = 17$ ms^{-1}, the rate of association of carbachol at the second binding site can be estimated as $k_2 = 5 \times 10^3$ ms^{-1} M^{-1} $= 5 \times 10^6$ s^{-1} M^{-1}. This is well below limits estimated for diffusion-limited association processes (Fersht, 1985).

Insights into the mechanism of receptor activation

The specific values of these rate and equilibrium constants provide some interesting insights into the mechanism of activation of the acetylcholine receptor by carbachol.

The first ligand binds tightly, but accelerates the rate of channel opening by only a factor of 40, and increases the channel mean open time by a factor of 5. The second ligand binds less tightly than the first, but accelerates the rate of channel opening by an additional factor of 2500. Binding of the second ligand increases the mean open time by another factor of 5.

The major effect of ligand binding is destabilization of the closed state as opposed to stabilization of the open state. This point has been made previously (Jackson, 1986*a*). It is evident that binding the second ligand destabilizes the closed state much more than does binding the first.

The dissociation constants for the binding of the first and second ligand to the receptor differ by a factor of only 4 when the channel is *open*. In contrast, the dissociation constants differ by a factor of at least 700 when the channel is closed. It would thus appear that the ligand–receptor interactions at the two binding sites are substantially more similar in strength when the channel is open than they are when the channel is closed. This difference between the binding sites has a direct parallel in the 'utilization' of binding energy by an enzyme. A good substrate may appear to bind weakly, only because much binding energy is utilized subsequent to binding to form an activated enzyme–substrate complex. This energy is then utilized to lower the activation energy for the enzyme-catalysed reaction (Fersht, 1985). Within this context, the binding energy of the second ligand is weaker, but more binding energy is available to push the channel into the open conformation. One can picture the second ligand binding site as a poor complement to the ligand when the channel is closed, forming incomplete contacts with the ligand. The ligand is thus more easily dislodged, but if the channel then opens, the binding site changes its configuration to complement better the structure of the ligand, and thus strengthen the ligand–receptor contacts. The idea of utilization of energy can also be invoked to explain differences between two populations of acetylcholine receptors recently observed by Auerbach & Lingle (1987). One population is activated at lower concentrations and opens more slowly than the other.

This picture might appear to suggest that ligand binding stabilizes the open state, in apparent conflict with interpretations of the ligand-induced changes in gating rates stated above. This can be reconciled by noting that ligand binding displaces bound water, which may be able to complement equally well the binding sites of either the open- or closed-receptor channels. Thus, it is the replacement of water by ligand that destabilizes the closed state.

With an estimate of $J_2 = 0{\cdot}11$ μM and a diffusion-limited upper bound to the rate of association of ligand with OA_1, an upper bound to $2j_{-2}$, the rate of dissociation of ligand from OA_2, is estimated as 0·04 ms^{-1}. This is slower than the rate of closure of a doubly liganded open receptor channel (0·2 ms^{-1}). Therefore, in most cases the channel closes first, then the ligand dissociates.

This reasoning may seem circular since R_2 and J_2 were calculated by assuming that $\nu_2 = \alpha_2$, and thus neglecting $2j_{-2}$. However, incorporating a non-zero value for $2j_{-1}$

into the calculation of R_2 lowers the maximum value calculated for $2j_{-2}$. Furthermore, since carbachol is not one of the best agonists, it is likely that the association rate, j_2, is well below the diffusion-limited maximum, strengthening this point.

A similar consideration allows one to estimate whether transitions to the doubly liganded receptor channel open state, OA_2, are most likely to occur from the doubly liganded receptor channel closed state, CA_2, rather than from the singly liganded receptor channel open state, OA_1. With the maximum rate of association of ligand to OA_1 limited by diffusion, at 5 μM-carbachol the rate of this process would then be 1·5 ms^{-1}. The mean open time of singly liganded receptors is 1 ms, suggesting that only if the rate of association is at its theoretical maximum, and nearly 70 times greater than the rate of association with CA_1, will even 60% of singly liganded receptors with open channels become doubly liganded before closing. Yet at 5 μM-carbachol, more than two-thirds of the openings are part of the long-duration component. Even at the maximum possible rate of association, some openings must appear from CA_2. As noted with the previous point, it is likely that with carbachol the rate of association is lower than the theoretical limit, in which case most openings would arise from CA_2.

Conclusion

Kinetic analysis of acetylcholine receptor channel gating provides detailed insight into the closed–open channel equilibria, the ligand binding equilibria, and how these processes are coupled. We see that in some respects the activation process fits the scheme proposed by Monod *et al.* (1965), with a detectable equilibrium for all states of occupancy of the receptor, and with the equilibrium shifting progressively with increasing numbers of ligand bound. On the other hand, the ligand binding sites are not equivalent, either in strength or in the degree to which they influence the channel equilibrium. Thus, the symmetry of the Monod *et al.* (1965) model is broken. The apparent negative co-operativity of the binding process is a feature often invoked in the activation process proposed by Koshland *et al.* (1966). However, this model does not provide for unliganded activation. With the level of detail provided here, it is probably not productive to attempt to force a process to satisfy the predictions of the classical theories. A more general theoretical framework is called for (Eigen, 1967).

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