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ACTIVATION OF ION CHANNELS IN THE FROG END-PLATE BY HIGH CONCENTRATIONS OF ACETYLCHOLINE

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SUMMARY

1. The equilibrium relationship between acetylcholine (ACh) concentration and response (fraction of channels open), corrected for the effects of desensitization, has been estimated by single-ion-channel recording at the adult frog skeletal neuromuscular junction. At high ACh concentration channel openings occur in well-defined clusters separated by long desensitized intervals. The response, p_o, was estimated as the proportion of time for which a single channel was open during a cluster.

2. At negative membrane potential (−120 mV) p_o reached a maximum value of 0·9 at 100 μM-ACh and was half-maximum at 15 μM with a Hill slope of 1·6 at this point. At concentrations higher than 200 μM-ACh, p_o declined as a result of open-channel block by free ACh itself.

3. At positive membrane potentials (+100 mV) there was little channel block by ACh; p_o reached a maximum value of 0·41 at 500 μM-ACh, with half-maximum activation at 50 μM and Hill slope of 1·2 at this point.

4. Particular mechanisms for channel activation by ACh were fitted to the data by the method of least squares. Fits were fully determinate only if the two binding sites for ACh were assumed to be equivalent with no co-operativity in the ACh binding reactions. At negative potential the microscopic equilibrium constant for binding was $K_1 = K_2 = 77$ μM and the equilibrium constant for channel opening (opening/closing rates, β/α) was 32. At positive potential the affinity was slightly higher, $K = 32$ μM, which confirms the view that the binding sites for ACh are outside the membrane electric field. The equilibrium constant for channel opening was reduced to 0·7 mainly as a result of the much shorter open lifetime (increased closing rate α) at positive potentials.

5. The data were also fitted well by very high values of β/α together with a high degree of negative co-operativity or non-equivalence in ACh binding affinity ($K_2 \gg K_1$). A good fit could also be obtained with moderate positive co-operativity combined with non-equivalence of the binding sites.

6. A mechanism that postulates a receptor with two independent gating subunits provided a poor fit to the data at negative potential.

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7. The rate constants for channel opening and ACh dissociation were estimated by constraining the fitted parameters so that the burst length for channel opening was equal to its observed value at low concentrations of ACh. The results were consistent with those of Colquhoun & Sakmann (1985) and support their interpretation of the fine structure of bursts at low ACh concentration. The rate constants estimated give high values for the ACh association and dissociation rates (8×10^7 M^{-1} s^{-1} and 6000 s^{-1}) and for the channel opening rate (20000 s^{-1}). These are similar to the values obtained by Colquhoun and Sakmann at low concentration.

INTRODUCTION

It is often, and properly, said that the nicotinic acetylcholine receptor is the most thoroughly characterized receptor-ion channel. It is also true that one of the most fundamental things that one would like to know about any drug receptor system is its equilibrium concentration–response relationship. It is, therefore, perhaps surprising that considerable doubt still exists about this relationship (for a review, see Colquhoun, Ogden & Cachelin, 1986). The first problem is to define exactly what concentration–response curve we are interested in: if genuine equilibrium is attained the curve will reflect desensitization and channel block, as well as channel activation. When the term 'equilibrium concentration–response relationship' is used it is usually intended to refer to the activation process only, and this is the sense in which the term will be used here. It is the relationship thus defined which can give information about the mechanism of ion channel activation, and hence the mechanism of synaptic transmission.

The biggest problem has always been desensitization (Katz & Thesleff, 1957) which is very profound. At the frog end-plate the equilibrium response to high agonist concentrations is only a few per cent of the peak response (e.g. Colquhoun *et al.* 1986). More recently ion channel block by the agonist itself has been discovered (Sine & Steinbach, 1984*b*; Ogden & Colquhoun, 1985). Many ingenious experiments have been done to try to avoid (e.g. by rapid agonist application) or to correct for, the effects of desensitization (Adams, 1975; Dionne, Steinbach & Stevens, 1978; Dreyer, Peper & Sterz, 1978; Lester & Nerbonne 1982; Pasquale, Takeyasu, Udgaonkar, Cash, Severski & Hess, 1983; Dilger & Adams, 1984). Despite the difficulty of some of these experiments, they have not led to unanimity. More recently single-channel methods have allowed more effective elimination of the effects of desensitization than was previously possible (Sakmann, Patlak & Neher, 1980). The methods available at that time did not permit the use of sufficiently large concentrations of agonist for saturation to be reached. In this paper we use a method based on that of Sakmann *et al.* (1980) to investigate the response to ACh over a wide range of concentrations and membrane potential. Some preliminary results have been published (Ogden & Colquhoun, 1983, 1985; Ogden, 1985).

METHODS

Twitch fibres of m. cutaneus pectoris, m. semitendinosus or m. sartorius of *Rana temporaria* were used. Connective tissue and presynaptic terminals were removed from the end-plate region by digestion with collagenase 0·2% (Sigma type 1a) and protease 0·02% (Sigma type 7) (Betz &

Sakmann, 1973). Ringer solution contained (mM): NaCl, 116·5; KCl, 2·5; $CaCl_2$, 1·5; HEPES, 10; pH 7·2; tetrodotoxin (Sankyo), 50 nM. Pipettes contained acetylcholine (ACh) dissolved in normal Ringer solution. During recording, fibres were depolarized by bathing in high-KCl Ringer solution containing 15 or 25 mM-KCl substituted for NaCl. This minimized movement of the fibre when a pipette containing high ACh concentration approached the end-plate region. Diffusion of ACh from the tip was also reduced by employing high-resistance (20–40 MΩ) patch pipettes made from borosilicate glass. Fibres were viewed with Nomarski optics and high-resolution patch clamp recordings were made in the cell-attached mode (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) from a point at, or very near to, the postsynaptic end-plate membrane. Membrane potential was measured with an intracellular microelectrode. Data were stored on FM tape.

The mean current during a record was measured by integration over a specified interval, either with an analogue integrator and light-pen recorder (Medelec FOR1004) or by numerical integration of the digitized record. For single-channel analysis the record was filtered at 3 kHz (−3 dB, 48 dB/octave Bessel) and a continuous section digitized at 32 or 40 kHz. An idealized record of amplitudes, open and gap times was obtained by direct fitting of transitions with the system response to a step input. A consistent time resolution was imposed on the data, usually 40 or 50 μs for shut periods and 80 or 100 μs for openings. Data were displayed as histograms of apparent open or closed times and fitted with probability density functions by the method of maximum likelihood (Colquhoun & Sigworth, 1983).

Curve fitting and assessment of errors

The equations for various possible reaction mechanisms were fitted to experimental data by a weighted least-squares criterion. A general minimization procedure was used to find the values of the parameters (e.g. equilibrium constants) that minimized the weighted sum of squared deviations,

$$S = \Sigma w_i(y_i - Y_i)^2,$$

where y_i is the mean of the observed values at the ith concentration, Y_i is the calculated value at this concentration, and the weight is $w_i = 1/s^2(y_i)$ where $s(y_i)$ is the observed standard error.

It is possible to calculate approximate standard errors for each of the parameter estimates (e.g. as described by Colquhoun, Dreyer & Sheridan, 1979, or Colquhoun & Sigworth, 1983). These provide a satisfactory indication of the range of uncertainty in linear cases (i.e. when the calculated response is a linear function of the parameters) or if the parameter estimates are rather precise. However, the present cases are all non-linear and some parameters are poorly determined, so standard errors are unsatisfactory. A much better way of specifying errors, which has been adopted here, is to calculate likelihood intervals (or support intervals; see Edwards, 1972, and Colquhoun & Sigworth, 1983). If the observed means have a Gaussian distribution then the log-likelihood, L, is

$$L = -S/2.$$

Thus, the least-squares estimates of the parameters, which minimize S, are the same as the maximum likelihood estimates, which maximize L, since $L_{max} = -S_{min}/2$. The most likely values of the parameters are those that correspond to L_{max}. The likelihood intervals for a particular parameter were found by holding that parameter at a fixed value (away from its maximum likelihood value) and repeating the fit with all other parameters free to vary. The maximum value of L that is achieved, L_{crit} say, will not be as large as that for the unconstrained fit. In order to find the 0·5-unit likelihood interval the fixed value is adjusted until a log-likelihood value of $L_{crit} = L_{max} - 0{\cdot}5$ is obtained. This value of L_{crit} can (normally) be obtained for two values of the parameter, one above and one below its maximum likelihood estimate; these upper and lower limits are usually unsymmetrically disposed relative to the best-fit value, whereas standard error estimates necessarily produce symmetrical limits. It is not possible to attach exact probabilities to likelihood intervals, but it may be noted that a 0·5-unit interval would correspond, in the case of linear problems, to plus or minus one standard error. When, as here, there is more than one parameter the errors for the parameters jointly might more properly be represented by a likelihood region (e.g. Colquhoun, 1979); the single-parameter limits represent tangents to this region, and are more convenient for the present purposes.

RESULTS

Experiments at negative membrane potentials

Single-channel currents recorded with high concentrations of ACh occur in clusters of high channel opening activity separated by long silent intervals. This is illustrated by a record obtained with 200 μM-ACh in the pipette at -116 mV shown in Fig. 1. Four clusters, of 500–4020 ms duration were observed, separated by silent intervals of 19·2–446 s (7·4 min) as indicated at the end of each trace. This behaviour of the nicotinic ion channel was described by Sakmann *et al.* (1980). The long silent periods are interpreted as times when all receptors in the patch are simultaneously desensitized. The durations of clusters are given for several ACh concentrations at -95 to -130 mV in Table 1. In the experiments reported here channel opening within clusters was studied as a function of ACh concentration in order to obtain information about nicotinic channel activation while eliminating, as far as possible, the effects of desensitization processes.

It was important for the interpretation of results that channel opening within those clusters studied was due to the activity of only one channel, despite the fact that histological evidence suggests that many hundreds should be present within the area of a patch on the postsynaptic membrane (Matthews-Bellinger & Salpeter, 1978). Recording sites were selected on the basis of a large extracellular current recorded with the patch pipette in close proximity to the membrane, due to leakage of ACh from the tip. With this procedure, sites of high activity were located to within 1–2 μm, so recordings were made from areas of high channel density on the postsynaptic membrane. Once a high-resistance seal was formed, isolated clusters rarely showed more than a single level of current amplitude at ACh concentrations greater than 5 μM. Those showing more than one level were excluded from analysis. Calculation shows that a double event would occur with 0·99 probability in fifty openings of a cluster of overall open probability 0·1 if two channels were independently active (see Colquhoun & Hawkes, 1983). In the experiments reported data were obtained at ACh concentrations greater than 2–5 μM in which the open probability was greater than 0·05. Since only one channel was active at a time, it may be supposed that others present were in long-lived desensitized states and unavailable for activation by ACh in the pipette. Furthermore, if the density of receptors in each patch were as high as expected, then each cluster would probably have arisen from the activity of a different channel.

The quantity of main interest here was the probability, denoted p_o, that a channel is open during a cluster (the open probability, for short). This is equivalent to the fraction of a population of identical, independent, non-desensitized channels in the open state. The value of p_o was estimated as the proportion of time for which the channels were open during clusters obtained over a wide range of ACh concentrations. Figure 2 shows records of clusters obtained at four agonist concentrations at -120 mV membrane potential. The upper trace in each panel shows inward current due to channel opening as downward deflections and the lower trace is the output of an integrator. This was used to calculate the mean current during a cluster from the ratio of total charge to cluster duration. As previously noted (Neher, 1983; Ogden & Colquhoun, 1985) with this procedure the mean current obtained is not reduced by

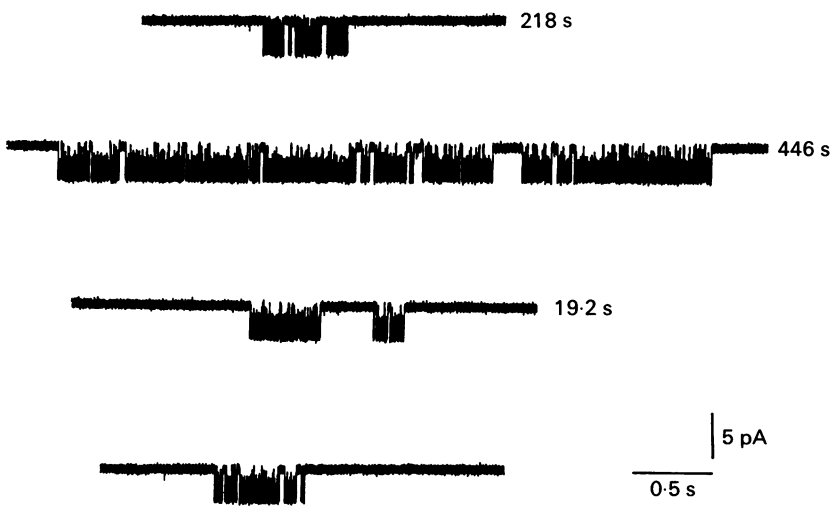


Fig. 1. Continuous record of single-channel currents recorded with 200 μM-ACh in the pipette solution. Potential −116 mV. Temperature 10–12 °C. Low-pass filter 3 kHz, −3 dB. Duration of silent intervals between clusters of openings given adjacent to each trace.

TABLE 1. Duration of clusters of openings and open probability within clusters at several ACh concentrations. Membrane potential −95 to −130 mV. Temperature 10–12 °C

Concentration (μM)	Cluster length mean ± S.D. (ms)	p_o mean	S.D.	S.E.M.	n
5	3910 ± 2200	0·09	0·044	0·018	6
10	876 ± 1180	0·38	0·174	0·041	25
20	836 ± 764	0·57	0·114	0·033	12
40	388 ± 387	0·67	0·119	0·036	11
50	141 ± 99	0·82	0·072	0·024	9
100	154 ± 73	0·87	0·092	0·018	26
200	193 ± 207	0·82	0·112	0·028	16
500	740 ± 590	0·70	0·056	0·015	14
1000	168 ± 92	0·55	0·065	0·038	3

low-pass filtering during recording. The proportion of time for which the channel was open was estimated as the ratio of mean current to the amplitude of the channel open level, measured directly from the current trace. At concentrations greater than 200 μM-ACh, the channel openings were so brief (as a result of channel block by ACh, see Ogden & Colquhoun, 1985) that few of them reached full amplitude. In these cases, the full current amplitude was obtained from data at low concentration under similar conditions. The error associated with this procedure is unlikely to be large because of the small variation of channel conductance from one fibre to another that is observed at the neuromuscular junction (Gardner, Ogden & Colquhoun, 1984). As may be seen in the records of Fig. 2, the open probability, p_o, within clusters increased from 0·09 in the record at 5 μM-ACh to 0·91 at 100 μM-ACh, and at 1 mM-ACh declined to 0·55.

The increase of p_o was associated with a reduction in the duration of gaps between

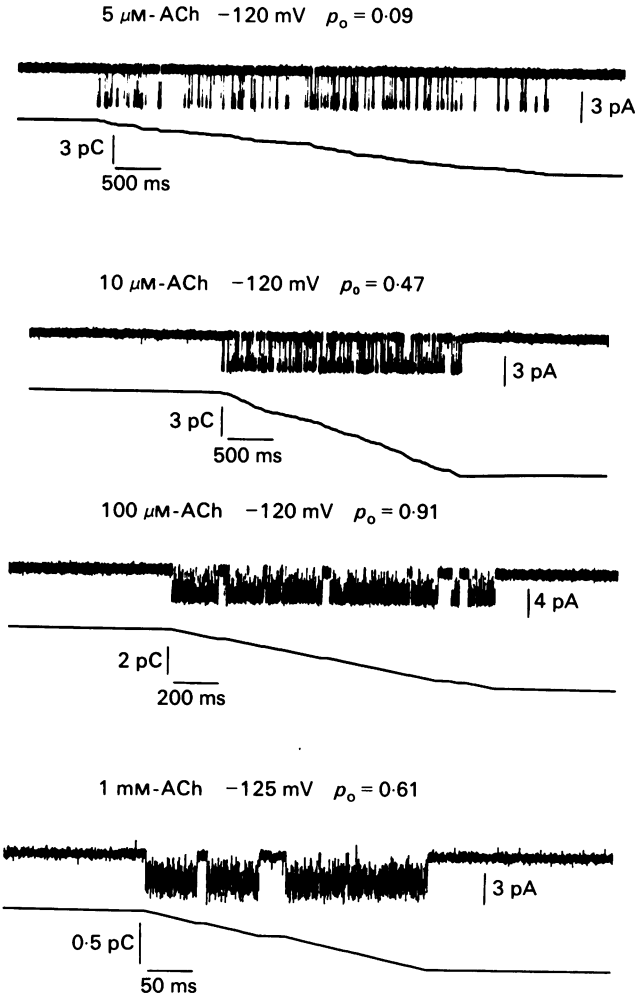


Fig. 2. Single clusters of unitary currents recorded with different concentrations of ACh in the pipette. ACh concentration and open probability given above each record. Upper trace: pipette current; lower trace: integral of pipette current. Mean currents calculated by dividing integral by cluster length; open probability by dividing mean current by full single-channel current amplitude. Potentials −120 to −125 mV. Temperature 10–12 °C. Low-pass filter 3 kHz (−3 dB).

openings. At ACh concentrations up to 50 μM, distributions of gaps within clusters were fitted well by the sum of three exponential components, one of mean about 20 μs and large amplitude due mainly to channel block in this range of concentrations (and partly to spontaneous shuttings, which also last about 20 μs), an intermediate component of mean 0·1–0·5 ms and very small amplitude, and a third component with mean duration which depended on ACh concentration, decreasing from 40 ms at 5 μM to 1 ms at 40 μM. The strong concentration dependence of the latter component suggests that it represents the time between individual channel activations (spent largely in the unliganded state). In this concentration range, gaps

within clusters more than five times longer than this 'mean time between activations' occurred with frequencies of less than 1 %. Gaps of this latter kind were probably not associated with the activation process but with fast receptor desensitization (Sakmann *et al.* 1980). At higher concentrations they occurred with greater frequency and would produce underestimates of p_o if included. Thus, at concentrations greater

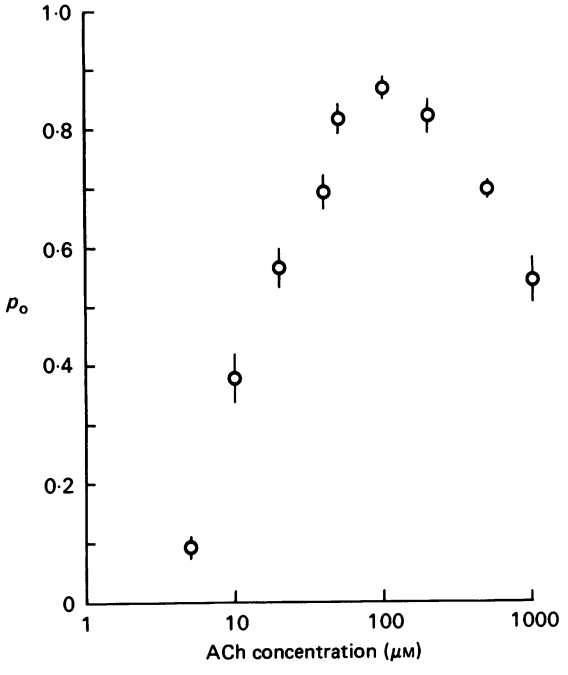


Fig. 3. Open probability, p_o, plotted against ACh concentration (log scale). Points are mean ± S.E.M. (vertical bars) of three to eighteen experiments. Potential −95 to −130 mV. Temperature 10–12 °C.

than 50 μM, gaps longer than 10 ms were excluded from analysis, resulting in subdivision of clusters into groups, and the mean current was determined within groups. Reference to the overall distribution of shut times suggests that this criterion would result, if anything, in a slight underestimation of p_o as a result of inclusion of some brief desensitized periods. Table 1 summarizes the mean durations of clusters (up to 50 μM-ACh) and groups within clusters from which p_o measurements were made.

The mean and standard error of p_o in eight to seventeen estimates at each ACh concentration are summarized in Table 1 and plotted in Fig. 3 against log concentration. This shows a steep increase of p_o in the range 5–50 μM-ACh with $p_o = 0{\cdot}5$ at about 15 μM, a maximum of $p_o = 0{\cdot}87$ at 100 μM, and then a decline of p_o with further increase of concentration above 200 μM to 0·55 at 1 mM. The steep rise of open probability at low concentrations when plotted as a Hill plot (see Fig. 8) is in agreement with membrane potential and conductance data obtained with low concentrations of ACh or ACh analogues (Jenkinson, 1960; Rang, 1971; Adams, 1975; Dionne *et al.* 1978; Dreyer *et al.* 1978). The decline of p_o at high concentration

has been described previously at the neuromuscular junction and may be attributed to block of the open ion channel by free ACh molecules (see Ogden & Colquhoun, 1985, for further discussion). The maximum level of $p_o \simeq 0{\cdot}9$ shows that at least 90% of available channels can be activated by high concentrations of ACh at the neuromuscular junction. However, the presence of block of open channels suggests that the true maximum for channel activation by ACh is probably much greater.

Experiments at positive membrane potentials

It is well known that block of open nicotinic ion channels by cationic organic compounds shows a marked reduction in affinity as the membrane potential is made more positive (Adams, 1977*b*; Neher & Steinbach, 1978). This has been shown to occur also with ACh in BC3H-1 cell line (Sine & Steinbach, 1984*b*) and at the neuromuscular junction (Ogden & Colquhoun, 1985). To obtain better definition of the maximum p_o, experiments were made at membrane potentials of +85 to +110 mV to minimize the blocking action of ACh and to investigate the potential dependence of the activation process.

At positive potentials and high concentrations, currents occurred in clusters separated by long silent intervals as at negative membrane potential. These are summarized in Table 2. Figure 4 shows two clusters recorded in the same membrane patch with 500 μM-ACh present in the pipette. The upper trace was recorded at a membrane potential of +85 mV, the lower a few seconds later after changing the potential to −95 mV. The mean duration of an opening is far shorter at positive potentials than at negative potentials, as expected (Magleby & Stevens, 1972). Inspection of the records at +85 mV shows that the currents were attenuated by low-pass filtering (in this case to an apparent maximum level of +1·7 pA at 4 kHz bandwidth). However, data obtained with 400 nM-ACh under the same conditions contained a sufficient number of full-amplitude openings to permit estimation of a mean value of +2·64 pA for the current amplitude, and a mean apparent open time of 90 μs. This amplitude suggests a channel conductance of 31 pS (assuming a reversal potential near 0 mV), which is the same as the value found in the negative range of potentials (Anderson & Stevens, 1973; Gardner *et al.* 1984). The apparent open time is in reasonable agreement with a value obtained from noise analysis by Adams, Nonner, Dwyer & Hille (1981) and is consistent with an e-fold increase of the channel closing rate in about 70 mV of depolarization, similar to values obtained in the negative range of potentials (Magleby & Stevens, 1972; Anderson & Stevens, 1973; Colquhoun & Sakmann, 1985). The reduced amplitude of currents at positive membrane potentials can therefore be attributed to attenuation of brief current pulses by low-pass filtering. At positive potentials the reduced apparent open time is unlikely to be due to open-channel block by the agonist since it occurs at both low and high agonist concentrations.

The mean currents and open probabilities during clusters were measured as described above and examples of clusters recorded at +100 mV at three ACh concentrations are illustrated in Fig. 5. The durations of clusters, summarized in Table 2, were similar to those observed at negative membrane potential, and cluster length decreased at high concentration of ACh. The mean p_o data (±S.E.M) are summarized in Table 2 and plotted against log ACh concentration in Fig. 6. In

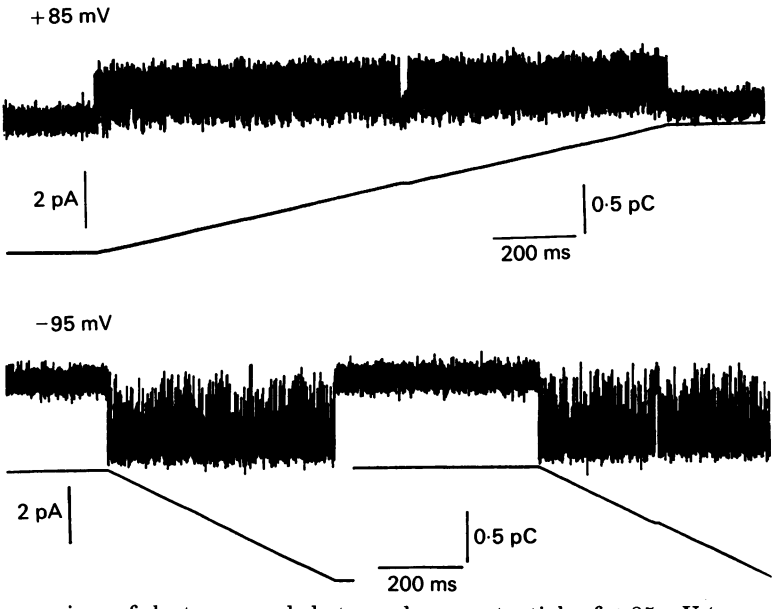


Fig. 4. Comparison of clusters recorded at membrane potentials of +85 mV (upper trace) and shortly after in the same patch at −95 mV (lower trace). 500 μM-ACh in the pipette. Temperature 12 °C. Low-pass filter 4 kHz (−3 dB).

TABLE 2. Duration of clusters and open probabilities at several ACh concentrations. Membrane potential range +85 to +120 mV. Temperature 10–12 °C

Concentration (μM)	Cluster length mean ± S.D. (ms)	p_o mean	S.D.	S.E.M.	n
10	2135 ± 1210	0·047	0·02	0·005	16
20	964 ± 786	0·085	0·02	0·006	11
50	1180 ± 661	0·19	0·05	0·020	6
100	608 ± 996	0·30	0·06	0·015	16
200	327 ± 363	0·33	0·07	0·014	25
500	529 ± 522	0·39	0·08	0·030	7
1000	532 ± 714	0·38	0·09	0·026	12
5000	311 ± 89	0·42	0·09	0·036	6
10000	74 ± 67	0·36	0·07	0·011	40

contrast with data at negative membrane potential, it can be seen that p_o increased more slowly to a maximum of 0·42 at 500 μM-ACh and declined only slightly at very high concentrations; the results look like a classical concentration–response curve for a partial agonist (see Discussion).

Interpretation of equilibrium concentration–response data

The open probability measured within clusters provides an estimate of the equilibrium occupancy of the open state of the non-desensitized channel. This open probability as a function of concentration, plotted in Figs 3 and 6, therefore provides a good representation of the equilibrium concentration–response relation, after

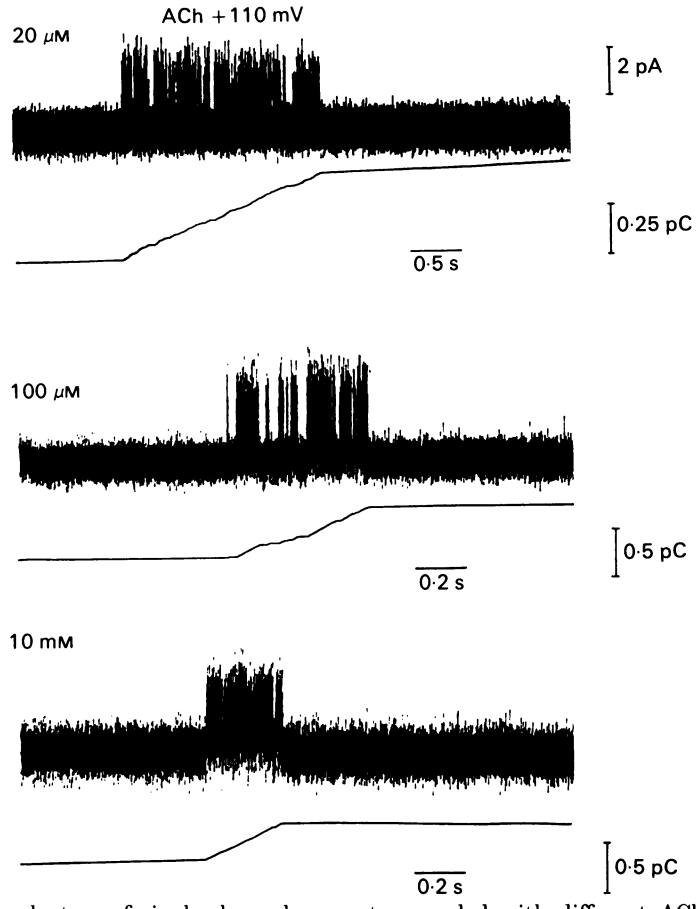


Fig. 5. Three clusters of single-channel currents recorded with different ACh concentrations present in the pipette as indicated above each trace. Membrane potential +100 mV. Temperature 10 °C. Low-pass filter 4 kHz (−3 dB).

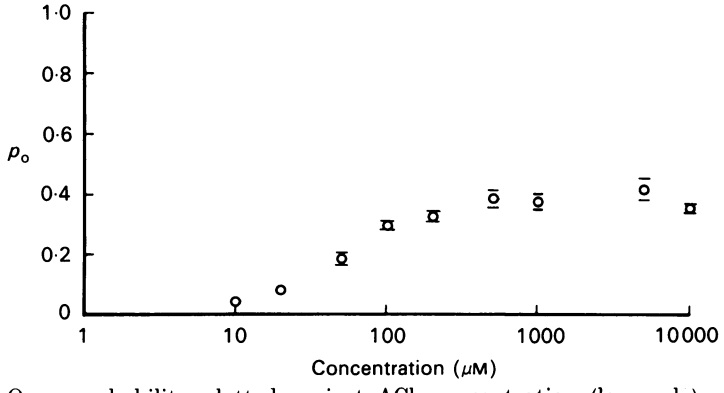


Fig. 6. Open probability plotted against ACh concentration (log scale) at membrane potentials +85 to +120 mV. Mean ± S.E.M (bars or symbol size).

elimination, as far as is possible, of the effects of desensitization. A comparison may be made between these data and the predictions of models for channel opening to obtain estimates of the equilibrium constants for the reactions involved.

The simplest plausible model for activation has sequential agonist binding and channel-opening steps (del Castillo & Katz, 1957). To account for the sigmoid rise of response with increasing concentration, and the Hill slope of greater than unity, two binding steps (at least) are required (Katz & Thesleff, 1957; Jenkinson, 1960; Rang, 1971; Magleby & Terrar, 1975; Adams, 1975; Dionne *et al.* 1978; Dreyer *et al.* 1978). The presence of two binding sites for ACh has been clearly demonstrated in structural studies of the nicotinic receptor (see Karlin, Cox, Kaldany, Lobel & Holtzman, 1983).

The conformation change resulting in channel opening occurs mainly in the doubly occupied channel. Although evidence obtained by Colquhoun & Sakmann (1985) suggests that brief openings of the singly-bound channel may occur at very low ACh concentrations, these were shown to decline sharply in frequency as concentration was increased and were found to occur with very low frequency at 5 μM-ACh and were not detected at higher concentrations in the present analysis. There is strong evidence to suggest that the decline of open probability seen with high ACh concentrations at -120 mV is due to a rapid block of the open channel by free ACh (Ogden & Colquhoun, 1985).

Three reaction schemes

Three reaction schemes were fitted to the data. The first has sequential binding of two ACh molecules, denoted A, to two equivalent sites, R, on the receptor, followed by opening and then block. It is represented by the scheme

$$\mathrm{R} \overset{\frac{1}{2}K_1}{\rightleftharpoons} \mathrm{AR} \overset{2K_2}{\rightleftharpoons} \mathrm{A_2R} \overset{\beta/\alpha}{\rightleftharpoons} \mathrm{A_2R^*} \overset{K_B}{\rightleftharpoons} \mathrm{A_2R^*A} \qquad \text{(S 1)}$$

in which R* represents the open channel and A_2R^*A the doubly liganded open channel blocked by an ACh molecule. K_1 and K_2 are microscopic equilibrium constants for the binding reactions (the subscripts indicate binding of the 1st and 2nd molecules respectively), β/α is the equilibrium constant for the channel opening reaction (opening/closing rate constants) and K_B is the equilibrium constant for block of the open channel by free agonist. In this scheme the order in which ACh binds to or dissociates from the sites is not specified (they are equivalent), but the possibility that an interaction between first and second binding steps can occur can be described by specifying different values for K_1 and K_2. The ratio K_1/K_2 is thus a measure of the inherent co-operativity in the binding reaction; the response, i.e. p_o, will, of course, show co-operativity with an initial Hill slope of 2 (declining to 1 at high concentrations) even when $K_1 = K_2$, i.e. when the binding steps do not interact. The occupancy of the open state at equilibrium for this scheme is given by the expression

$$p_o = \frac{c_1 c_2 \beta/\alpha}{1 + 2c_1 + c_1 c_2(1 + \beta/\alpha) + c_B c_1 c_2 \beta/\alpha}, \qquad (1)$$

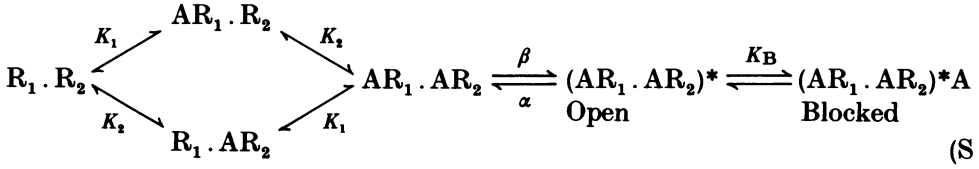
where c_1, c_2 and c_B denote the agonist concentration divided by the microscopic equilibrium constants for first and second bindings and for open-channel block by the

agonist, respectively. The slope n_H of the Hill plot for this scheme in the absence of channel block is

$$n_H = 2\left(\frac{1+c_1}{1+2c_1}\right). \tag{2}$$

At low concentration $n_H = 2$, and it declines to $n_H = 1$ at high concentrations; it is independent of both K_2 and β/α. The presence of open-channel block introduces a slope of n_H of -1 at very high concentrations (when $c_B \gg 1$; Ogden & Colquhoun, 1985).

The second scheme considered here arises from biochemical evidence that the two binding sites have inherently different affinities for some ligands, possibly including ACh (Weiland & Taylor, 1979; Sine & Taylor, 1981). The binding sites are non-equivalent in the sense that two distinguishable mono-liganded forms exist. The model for non-equivalent subunits can be written as the scheme



(S 2)

where K_1, K_2, β/α and K_B are microscopic equilibrium constants as before, $(AR_1.AR_2)^*$ is the open-channel conformation and $(AR_1.AR_2)^*.A$ is the open channel blocked by free ACh. It should be noted that the subscripts 1 and 2 have a quite different significance in scheme (S 2) from that in scheme (S 1). The ratio K_1/K_2 measures the extent of non-equivalence of the two sites in scheme (S 2); which site is numbered 1, and which 2, is therefore entirely arbitrary. This version assumes that the affinity for the binding to subunit type 1 is the same whether subunit type 2 is occupied or not (and vice versa), i.e. it assumes that there is inherent co-operativity in the binding reactions. The equilibrium occupancy of the open state is given by

$$p_o = \frac{c_1 c_2 \beta/\alpha}{1+c_1+c_2+c_1 c_2(1+\beta/\alpha)+c_B c_1 c_2 \beta/\alpha}, \tag{3}$$

where c_1, c_2 and c_B represent the ACh concentration divided by the equilibrium constants K_1, K_2 and K_B respectively.

Schemes (S 1) and (S 2) both have four arbitrary parameters to be estimated from the observations. If we constrain K_1 to be equal to K_2, then the two schemes become the same (and the number of parameters is reduced to three). The parameters can be chosen as K_1, K_2, β/α and K_B, or, if we wish to estimate the uncertainty in the relative values of K_1 and K_2, they can be chosen as K_1, K_2/K_1, β/α and K_B. Both approaches have been used to obtain the results in Tables 3 and 4. The slope of the Hill plot for this scheme, in the absence of channel block, is given by

$$n_H = 2\left(\frac{2+c_1+c_2}{2(1+c_1+c_2)}\right). \tag{4}$$

Again n_H falls from 2 at low concentration to 1 at high concentration.

A third possible mechanism can be envisaged along the lines of the Hodgkin–

Huxley postulate for the sodium channel mechanism. Suppose that the receptor contains two entirely independent (but not necessarily identical) subunits each of which can bind one agonist molecule, and then change its conformation according to the mechanism of del Castillo & Katz (1957). Suppose also that the channel is open only when both subunits are in the altered conformation (denoted by the asterisk). The scheme can be written, in the absence of block, as

$$\left.\begin{array}{l} R_1 \overset{K_1}{\rightleftharpoons} AR_1 \overset{\beta_1/\alpha_1}{\rightleftharpoons} AR_1^* \\ R_2 \overset{K_2}{\rightleftharpoons} AR_2 \overset{\beta_2/\alpha_2}{\rightleftharpoons} AR_2^* \end{array}\right\} \qquad \text{(S3)}$$

The open state (AR_1^*, AR_2^*) can then be blocked with equilibrium constant K_B, as before. It was found (see below) that the best fit was obtained on the assumption that the two subunits are identical (so $K_1 = K_2 = K$, c = concentration/K, and $\beta/\alpha = \beta_1/\alpha_1 = \beta_2/\alpha_2$); in this case

$$p_o = \frac{(c\beta/\alpha)^2}{[1+c(1+\beta/\alpha)]^2 + (c\beta/\alpha)^2 c_B}, \qquad (5)$$

and the Hill slope in the absence of channel block is

$$n_H = 2\frac{(1+c(1+\beta/\alpha))}{1+2c(1+\beta/\alpha))}, \qquad (6)$$

which, as in the other models, falls from 2 at low concentration to 1 at high concentration.

Results of fitting p_o curves

Sequential binding. Table 3 gives the least-squares estimates, and their likelihood intervals (or support intervals, see Methods), for fits to the p_o curve found at negative membrane potentials. The only scheme for which all the parameters can be estimated is that with $K_1 = K_2$. The microscopic equilibrium constant for binding is 77 μM, and $\beta/\alpha = 32$ is quite large (with limits 22–50). The equilibrium constant for block of open ion channels was about 1·3 mM; this was the most precisely determined value for all models. These values are compared below with those estimated by other methods.

When the assumption that $K_1 = K_2$ is relaxed, a somewhat better fit is obtained, the value of S_{min} being reduced from 14·1 to 13·2 (for either scheme (S 1) or (S 2)). This improvement in fit is slight, as may be seen by comparing the curves plotted with the mean data points in Fig. 7*A* or as judged by an approximate likelihood ratio test, or partial F test (see, for example, Colquhoun, 1979; Horn & Vandenberg, 1984). Even though our data are not precise enough to show clearly that K_1 and K_2 differ it is nevertheless desirable to consider how our conclusions would be affected if they did in fact differ. None of the models are entirely determined when we do not assume that K_1 and K_2 are equal; nevertheless the use of likelihood intervals allows useful limits to be placed on the values of the parameters. In particular, if either model (S 1) or (S 2) were true then neither β/α nor K_2 separately would be well defined, but nevertheless we can say that β/α is unlikely to be less than 25 (though it might have any value larger than this – the upper limit is effectively infinite). This lower limit for β/α is similar to the lower limit (22) found when we assume $K_1 = K_2$. Thus,

whichever model is assumed, it is likely that acetylcholine at high concentration could open at least 96% (i.e. $p_{o,max} = (\beta/\alpha)/(1+\beta/\alpha) = 22/(1+22)$) of ion channels, if there were no channel block or desensitization. It may be noted that the use of approximate standard errors for parameters instead of the likelihood intervals used here (see Methods) would not have allowed any useful conclusion to be drawn from these fits.

TABLE 3. Parameter estimates from data at membrane potentials of −95 to −130 mV

K_1 (μM)	K_2 (μM)	K_2/K_1	β/α	K_B (mM)	S_{min}
(a) Equivalent binding sites with $K_1 = K_2$ (scheme (S 1))					
77 (60–102)	[77]	[1]	32 (22–50)	1·2 (1·1–1·3)	14·1
(b) Equivalent binding sites (scheme (S 1))					
47 (19–91)	3700 (52–ind.)	79 (0·78–ind.)	1100 (25–ind.)	1·2 (1·1–1·3)	13·2
(c) Non-equivalent binding sites (scheme (S 2))					
24 (19–39)	3400 (80–ind.)	142 (1·0–ind.)	500 (25–ind.)	1·3 (1·1–1·4)	13·2
(d) Independent identical subunits (scheme (S 3))					
$1·6 \times 10^5$	[$1·6 \times 10^5$]	[1]	2300	1·3	41·0

The best estimates are given, with their 0·5-unit likelihood intervals in parentheses. A limit is specified as indeterminate (ind.) if no value can be found which reduces L by as much as 0·5. The minimum value, S_{min}, of the weighted sum of squared deviations is given in the last column. Values in square brackets are determined *ex. hypothesi*.

The estimates of K_2 and β/α were found to have a strong positive correlation, i.e. the effective equilibrium constant for the second binding plus opening, $K_2/(1+\beta/\alpha)$, is better defined than either parameter separately. This is hardly surprising because it is well known that for efficacious (large β/α) agonists the effect of increasing β/α ('efficacy') is indistinguishable from the effect of increasing affinity (e.g. decreasing K_2) – both cause a parallel shift to the left of the log(concentration)–response curve. This effect is illustrated for the present problem by Ogden, Colquhoun & Marshall (1987). As in the case of β/α, lower limits only could be defined for K_2, and for the ratio K_2/K_1 (see Table 3). The fit of scheme (S 1) suggests that the data are compatible with (but do not demonstrate) inherent negative co-operativity in the binding, the second binding step becoming lower affinity (compensated by large β/α) than the first. The fit of scheme (S 2) shows that the data are compatible with (but do not demonstrate) a large degree of non-equivalence of the two binding sites (though again only if β/α is large). Figure 7*A* shows the best-fit curves obtained with $K_1 = K_2$ constrained (curve 1) and unconstrained for sequential binding or non-equivalent sites (both represented by curve 2); as may be seen, the fits to the mean data points are equally good. The same curve and data points are shown in Fig. 7*B* with calculated curves for the open probability in the absence of open-channel block by the agonist (A) and for the occupancy of ACh activation sites (B).

The data are plotted as a Hill plot in Fig. 8. The slope at 50% of the maximum p_o value (i.e. at an ACh concentration of 16·3 μM) is 1·7, as expected from eqn (2) for

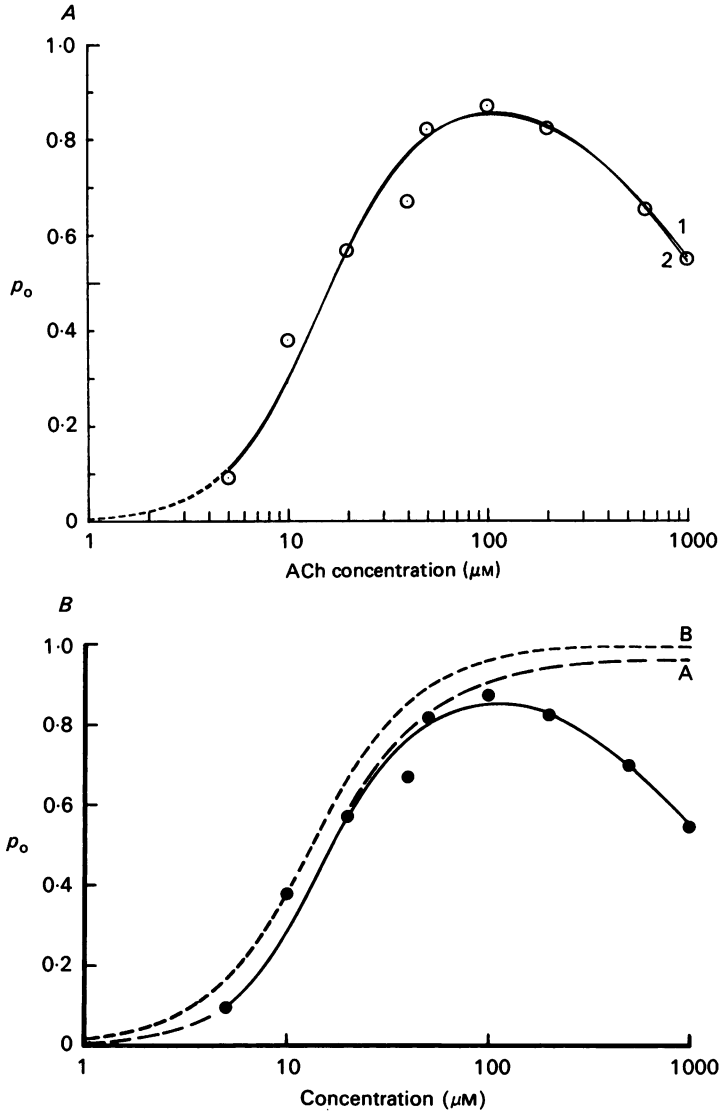


Fig. 7. *A*, best-fit least-squares curves for p_o as a function of ACh concentration (log scale) plotted with mean data points at −95 to −130 mV membrane potential. Curve 1: scheme (S 1) or (S 2) constrained with $K_1 = K_2$. Minimum sum of squares = 14·1. Curve 2: scheme (S 1) (co-operative binding) or (S 2) (non-equivalent subunits) with K_1 and K_2 independent. Minimum sum of squares = 13·2. *B*, best-fit curve for p_o (continous line), p_o calculated with same parameters without open-channel block (long dashes, curve A) and calculated occupancy of all ACh sites (short dashes, curve B) plotted with mean data points against ACh concentration (log scale).

the fit with $K_1 = K_2$; the other fits are similar. The effect of either negative co-operativity in binding, or of non-equivalence of the binding sites, is to reduce the slope of the p_o curve; the increased β/α value found in these two cases is needed to restore the slope to a value that fits the data ($n_H \simeq 1{\cdot}7$). If we consider a combination of schemes (S 1) and (S 2) in which both co-operativity and non-equivalence are

allowed, and we fix β/α at 32, then a fit very similar to those already obtained can be found with a tenfold non-equivalence of the binding sites combined with a 2-fold positive co-operativity of binding. The results are, therefore, also compatible with the view that a slight positive co-operativity in the binding cancels the effect of non-equivalence on the slope of the p_o relationship, the value of β/α being in the range

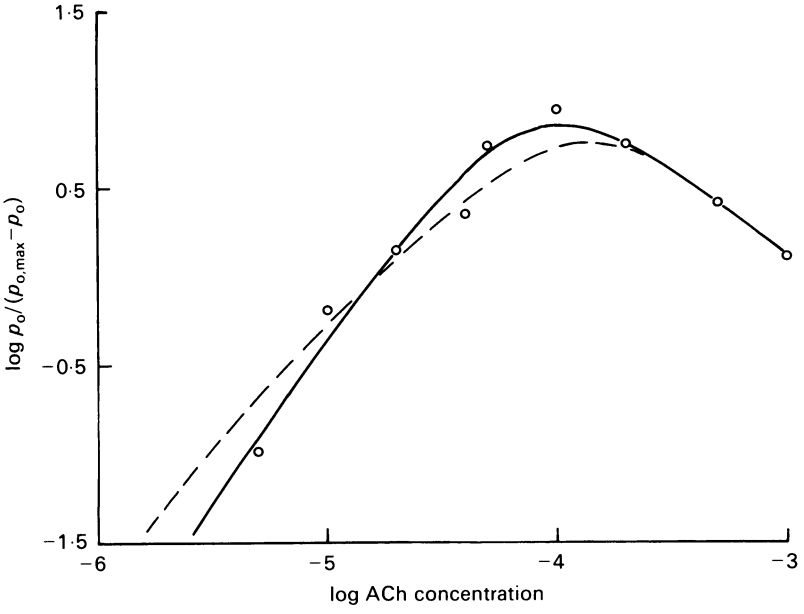


Fig. 8. Hill plot of log $[p_o/(p_{o,max}-p_o)]$ against log ACh concentration. Symbols: mean data points at −95 to −130 mV. Continuous curve: best-fit parameters for schemes (S 1) and (S 2). Dashed curve: best fit for independent-subunits scheme (S 3).

expected from low-concentration experiments, although it should be noted that the parameter values for this model are not well defined.

Independent subunits (scheme S 3). This mechanism did not fit nearly as well as the others. The results are shown in Table 3. The best fit was found when the subunits were supposed to be identical, a condition that made the curve as steep as possible. Only the affinity for channel block was well defined (1·3 mM as in other fits). Both K and β/α were very large, but their separate values were not well defined; essentially only their ratio, the effective equilibrium constant $K/(1+\beta/\alpha) \simeq 7$ μM, was well defined. However, even the best fit with the independent-subunit schemes was far worse than the best fits obtained with the other schemes ($S_{min} = 41{\cdot}0$ as against 14·1 or 13·2). Thus the results at negative potentials seem to be clearly inconsistent with the independent-subunit mechanism. The reason for this is that the curve cannot be made steep enough to provide a good fit to the results. Although all three schemes have a Hill slope (n_H) of 2 at low concentrations n_H falls more rapidly for the independent subunit case than for the others. For schemes (S 1) and (S 2) the predicted Hill slope at $p_o = p_{o,max}/2$ is 1·6–1·7 which fits the observations well, but for scheme (S 3) the Hill slope at this point is only 1·17. The fitted curves for schemes (S 1), (S 2) and (S 3) are presented as Hill plots with the mean data points for comparison in Fig. 8.

Data at positive membrane potentials

As would be expected, open-channel block is far less prominent at positive than at negative membrane potentials; very little decline of p_o is seen at high agonist concentrations (Fig. 6) and the equilibrium constant for block is estimated to be

TABLE 4. Parameter estimates from data at membrane potentials of +85 to +120 mV

K_1 (μM)	K_2 (μM)	K_2/K_1	β/α	K_B (mM)	S_{min}
(a) Equivalent binding sites with $K_1 = K_2$ (scheme (S 1))					
32 (30–35)	[32]	[1]	0·69 (0·65–0·73)	33 (23–54)	8·7
(b) Equivalent binding sites (scheme (S 1))					
16 (4–25)	46 (33–59)	2·9 (1·1–7·5)	0·75 (0·68–0·83)	23 (17–38)	7·4
(c) Non-equivalent binding sites (scheme (S 2))					
9·3 (4–25)	81 (45–115)	8·7 (1·6–27·6)	0·75 (0·68–0·83)	23 (17–38)	7·4
(d) Independent identical subunits (scheme (S 3))					
64 (59–71)	[64]	[1]	1·84 (1·75–1·94)	25 (20–40)	7·7

The values are specified as in Table 3.

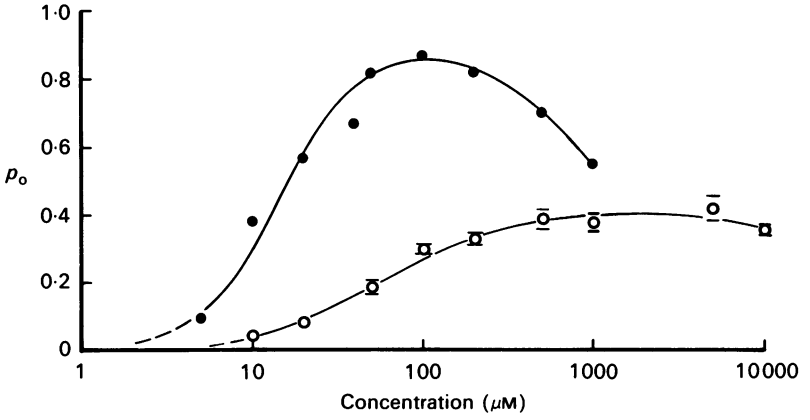


Fig. 9. Comparison of p_o data plotted against ACh concentration (log scale) at −95 to −130 mV (mean data, filled symbols) and +85 to +120 mV (mean ± S.E.M., open symbols). Best-fit curves for sequential schemes (S 1) or (S 2) are drawn through the points.

greater than 20 mM, whichever model is fitted (Table 4). This value corresponds to an e-fold reduction of blocking affinity per 70 mV of depolarization (Ogden & Colquhoun, 1985). It is assumed that the same activation schemes apply as at negative membrane potential and the opening of singly occupied channels has been neglected as before, although this point could not be examined in detail because of the short open lifetime at positive potentials.

The maximum response (p_o) is more clearly defined (Fig. 6), and is well below 1, so conditions are much more favourable than at negative potentials for the separate

estimation of β/α and K_2. The value of β/α was about 0·7 whichever model was fitted (roughly 50-fold smaller than at negative potentials), so the maximum fraction of channels that could be opened at +100 mV would be 41% in the absence of desensitization and channel block.

The equilibrium constant for binding, under the assumption that $K_1 = K_2$, is about 32 μM, slightly over twice the affinity at negative potentials. The voltage dependence of binding therefore seems to be far less pronounced than that of the mean open time, or of channel block; there appears to be an e-fold increase in binding affinity for about 250 mV depolarization. As in the case of data at negative potentials, relaxation of the assumption that $K_1 = K_2$ leads to a fit that is not demonstrably better, but again it is advisable to consider such fits because it is not improbable that the binding sites may in fact interact and/or be non-equivalent. As in the case of negative potentials, the result of fitting scheme (S 1) is consistent with a slight (3-fold) inherent negative co-operativity in the binding steps (taken in isolation); the fit to scheme (S 2) is consistent with a substantial (9-fold) degree of non-equivalence. However, in both cases the 0·5-unit likelihood intervals (roughly equivalent to $\pm$ one standard deviation, see Methods) indicate the results are also consistent with a ratio of K_2/K_1 close to unity. Figure 9 shows the best-fit curve obtained with $K_1 = K_2$ in scheme (S 1) or (S 2) plotted with the mean data points and for comparison the mean data points and best-fit curve obtained at −120 mV.

The slope, n_H, of the Hill plot at 50% of the maximum p_o value (i.e. at a concentration of 50·0 μM) is 1·24 for the fit with $K_1 = K_2$. For the other two fits we find $n_H = 1{\cdot}12$ at 50% maximum (at 60 μM). If we allow both non-equivalence and co-operativity then the results are found to be consistent with a slight (1·8-fold) degree of positive co-operativity in binding (which will cancel the effect of the former in reducing n_H). This conclusion is similar to that reached at negative membrane potentials.

Independent subunits. The results given in Table 4 show that at positive membrane potentials the independent-subunit mechanism (S 3) fits the results as well as the other models, contrary to what was found at negative potentials. This happens because, as pointed out above, the Hill slope of the data at positive potentials is much shallower than at negative potentials. The equilibrium constant for binding was 64 μM for each subunit, and the conformational equilibrium constant, β/α, was 1·84, so the predicted maximum response was $(1{\cdot}84/2{\cdot}84)^2 = 0{\cdot}42$.

Fits that incorporate knowledge of the low-concentration burst length

The results given above provide estimates of the equilibrium constants for ACh binding to receptors and for the channel-opening reaction and therefore give information only of the ratios of the rate constants for the forward and backward reactions. On the other hand, Colquhoun & Sakmann (1985) have made measurements of the kinetics of channel opening at low concentrations of ACh and have interpreted them in terms of the activation schemes given here to provide estimates of the rate constants associated with channel opening and closing and the dissociation of ACh. In doing the fitting it therefore seems sensible to introduce some kinetic information by taking into account the known value of the burst length of channel openings associated with a single activation (see below). The burst length is a directly

observable quantity and its use introduces no assumptions about the existence, detectability, or mechanism of interruptions that may occur during a single-channel activation.

Colquhoun & Sakmann (1985) observed brief shut periods (mean duration 20 μs) interrupting the channel opening produced by low concentrations of ACh. The event produced by a single activation of the receptor by ACh was therefore described as a burst of openings. They found (at a membrane potential of -130 mV) that a burst consisted, on average, of 2·9 openings, each of mean duration 1·4 ms, so the mean length of the burst was 4·2 ms. Very similar mean values have been observed at low ACh concentration in the present work: short gap duration 17 μs with 4·4 openings of 1·3 ms in long bursts of 5·8 ms duration. If these results are interpreted in terms of oscillation between A_2R and A_2R^*, according to scheme (S 1) or scheme (S 2), then values for the dissociation rate constant, k_{-2}, for the channel opening rate constant, β, and for the shutting rate constant, α, can be estimated. For example scheme (S 1) can be written

$$R \underset{k_{-1}}{\overset{2k_{+2}}{\rightleftharpoons}} AR \underset{2k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} A_2R \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} A_2R^*, \qquad \text{(S 4)}$$

where $K_2 = k_{-2}/k_{+2}$ and $K_1 = k_{-1}/k_{+1}$. Colquhoun and Sakmann found, in this way, $\beta = 30600\ s^{-1}$ and $k_{-2} = 8150\ s^{-1}$ (Table 5a). It is of interest to consider whether the present results can cast any light on the correctness or otherwise of their interpretation, since alternative explanation have been proposed for observations showing some similarities in the nicotinic receptor of a cell line (BC3H-1, Sine & Steinbach, 1986). The equilibrium models fitted so far can, of course, give no information about the rate constants. However, the fits to the p_o data can be constrained so that the mean burst length must have its observed value. The mean burst length at low agonist concentrations was 4·2 ms at -130 mV, and this value should not depend on concentration (provided a burst is defined as $A_2R \rightleftharpoons A_2R^*$ oscillations). All the suggested mechanisms imply that channel re-opening (oscillation) can, in principle, occur, but use of this sort of constrained fit introduces no assumptions whatsoever concerning whether the brief shut periods that are actually observed originate from such oscillations, or whether they have some quite different origin (the shut periods introduced by oscillations being, perhaps, too rare or too brief to be seen at all).

In the scheme (S 4), the mean number of openings per burst is

$$n_o = 1 + \beta/2k_{-2}, \qquad (7)$$

the mean duration of shut periods within bursts is

$$\mu_s = 1/(\beta + 2k_{-2}), \qquad (8)$$

and the mean duration of individual openings is

$$\mu_o = 1/\alpha. \qquad (9)$$

The mean burst length, μ_b, is given by the sum of the mean open and shut times per burst, viz.

$$\mu_b = n_o\mu_o + (n_o - 1)\mu_s, \qquad (10)$$

(see, for example, Colquhoun & Sakmann, 1981). The fits were done on the assumption that $k_{-1} = k_{-2}$ and $k_{+1} = k_{+2}$ (so $K_1 = K_2$). The parameters estimated

TABLE 5. Comparison of burst characteristics calculated by curve fitting with those observed at low ACh concentration

(a) Burst characteristics at −130 mV, 100 nM [ACh] (Colquhoun & Sakmann, 1985)

Mean burst duration (ms)	Mean no. of openings per burst	Mean gap duration (μs)	Mean open duration (ms)	β (s^{-1})	k_{-2} (s^{-1})
4·2	2·9	20	1·4	30 600	8150

(b) Results of curve fitting with $K_1 = K_2$ (mean burst length constrained to 4·2 ms)

$k_{+2}(=k_{+1})$ ($M^{-1}s^{-1}$)	S_{min}	Mean no. of openings per burst	Mean gap duration (μs)	Mean open duration (ms)	β (s^{-1})	k_{-2} ($=k_{-1}$) (s^{-1})
4×10^7	18·7	$1{\cdot}8\times10^5$	0·0013	$2{\cdot}3\times10^{-5}$	$7{\cdot}9\times10^8$	2209
6×10^7	14·11	6·2	17·5	0·68	47 840 (20400 –ind.)	4599 (4118 –5122
7×10^7	14·11	3·5	26·7	1·20	26780 (14400 –84000)	5360 (4215 –7194)
8×10^7	14·11	2·6	30·9	1·59	20131 (11800 –47600)	6116 (4820 –8210)
1×10^8	14·11	1·98	32·9	2·12	15052 (9430 –29700)	7654 (6030 –10280)
5×10^8	14·11	1·11	11·8	3·88	8358 (5760 –13600)	38280 (30130 –51430)
(c) Free fit of k_{+2} ($=k_{+1}$)						
$8{\cdot}9\times10^7$ ($6{\cdot}4\times10^7$ -2×10^8)	14·11	2·3	32·0	1·85	17220 (5304 –ind.)	6832 (2740 –ind.)
(d) Equivalent sites, K_1, K_2 not constrained						K_2/K_1
$1{\cdot}7\times10^8$	13·3	1·2	1·0	3·63	130000	35·0
(e) Non-equivalent sites						
$2{\cdot}2\times10^8$	13·3	1·1	0·8	3·96	68000	73·0

Values in parentheses are 0·5-unit likelihood intervals. A limit is specified as indeterminate (ind.) if no value can be found which reduces L by as much as 0·5.

were β, k_{-2} and K_B (rather than β/α, K_2 and K_B as before). The values of β and k_{-2}, in conjunction with μ_b, allow a calculation of α from eqns (7)–(10), and hence β/α is obtained. Postulation of a value of the association rate constant, $k_{+1} = k_{+2}$, or treating k_{+2} as another parameter to be estimated, allows calculation of $K_2 = K_1$ from the estimated k_{-2} value. Table 5b shows the best fits obtainable with $\mu_b = 4{\cdot}2$ ms and k_{+2} fixed within the plausible range of values 4×10^7 to 5×10^8 M^{-1} s^{-1}; the Table also shows the predicted burst characteristics calculated from eqns (7)–(10). Table 5c shows the result of a free fit with k_{+2} taken as one of the parameters to be estimated.

The free fit gives values of β and k_{-2} that are close to the values estimated, by a quite different argument, by Colquhoun & Sakmann (1985); the two estimates differ by a factor of less than two. The value of the association rate constant, $8{\cdot}9 \times 10^7$ M^{-1} s^{-1} (limits $6{\cdot}5$–20×10^7 M^{-1} s^{-1}) is exactly in the range expected for diffusion-limited access to the receptors. The lower limits for β and k_{-2} are well-defined but no upper limit can be found; the data are consistent, from a statistical point of view, with indefinitely large values of these rate constants. However, inspection of the results of fits with fixed k_{+2} values in Table 5b shows that the range of uncertainty is found to be much smaller than is suggested by these statistical limits when physical and experimental constraints are taken into account. Values for the association rate constant, k_{+2}, of anything much greater than 10^8 M^{-1} s^{-1} are physically implausible (e.g. Gutfreund, 1972) so values of β much less than 8000 s^{-1} are unlikely. Furthermore values of k_{+2} much smaller than 7×10^7 M^{-1} s^{-1}, although perfectly plausible from a physical point of view, are seen to fit the results only if we postulate that both β and α are very large so that a channel activation would consist of a large number of very short openings. If this were the case the channel openings should be very noisy, but this is not observed; there is only a small amount of extra noise while the channel is open at the frog end-plate at low ACh concentration (unpublished observations; see also Sigworth, 1985). It might be argued that individual openings are so brief (and numerous) that most of the excess noise is outside the observable bandwidth, but if this were the case it would be expected that the frequency and duration of openings and, therefore, the apparent single-channel conductance would depend on the nature of the agonist (which is not the case at the frog end-plate; Gardner *et al.* 1984), and quite possibly on membrane potential too (which is also not the case; the single-channel conductance was found to be 30 pS at +100 mV, the same as at negative potentials). The range of parameter values that is consistent with experimental results is thus restricted approximately to those in Table 5b that correspond to $k_{+2} = 6 \times 10^7$ to 10^8 M^{-1} s^{-1}. These values are exactly in the range predicted according to the interpretation placed on the fine structure of channel activation by Colquhoun & Sakmann (1985).

Removing the constraint $K_1 = K_2$ also produces a good fit to the data, achieved by introducing a high degree of negative co-operativity between equivalent sites (Table 5d) or non-equivalence of affinity for the two sites (Table 5e). With a burst length of 4·2 ms, both β and k_{-2} can take large values and the value of the mean open time, $1/\alpha$, approaches the burst length. However, this results in rather few (0·05–0·15) and very brief (1 μs) short gaps within bursts when compared with the observed frequency and duration of short gaps at low concentration (of 1·9 and 20 μs, respectively). Thus, although the high values of β/α associated with negative co-operativity or non-equivalence are consistent with the concentration–p_o data, the infrequent and very brief gaps within bursts predicted a low concentration are not consistent with the interpretation suggested by Colquhoun and Sakmann.

DISCUSSION

Acetylcholine as a transmitter

The results reported here suggest that high concentrations of acetylcholine would be able to open a large proportion, 97%, of ion channels at the neuromuscular junction if it were not for the effects of open-channel block by ACh itself and of desensitization, although the microscopic affinity for its binding to receptors is low ($\simeq 77\ \mu$M). These attributes are exactly what is needed for an efficient fast neurotransmitter. ACh molecules will bind rapidly because the transmitter concentration is high in the synaptic cleft, most channels will open very soon after binding (large opening rate constant, β) and ACh will dissociate rapidly after a few openings have occurred in quick succession (see, for example, Colquhoun & Sakmann, 1983, 1985). Even the high concentration of ACh in the synaptic cleft will produce little open-channel block (especially as such block becomes weaker as the postsynaptic membrane depolarizes) and the transmitter is not normally present for a sufficiently long time to produce any noticeable desensitization (but see Magleby & Palotta, 1981). Thus, the characteristics of ACh action which we have attempted to assess appear to be ideally suited to its physiological function as a fast synaptic transmitter.

Comparison with results at low concentration

The results are also of interest for the light which they can cast on the interpretation of the brief interruptions that can be seen during activations of the ion channel by low ACh concentrations (Colquhoun & Sakmann, 1981, 1985). The results found here are consistent with the proposal that these interruptions represent oscillations between the shut and open forms of the doubly occupied ion channel, i.e. repeated openings of the channel while it is occupied. This interpretation implies that the rate constant for ACh dissociation is not, as is often assumed, much faster than the channel opening reaction (Magleby & Stevens, 1972; Anderson & Stevens, 1973) but that both reactions occur at comparable rates. However, ACh is not the ideal agonist for a quantitative test of this hypothesis because of its high efficiency at opening ion channels. The results at low concentrations suggested that ACh would be capable of opening 98% of channels at high concentration (at -130 mV, 10–12 °C) and the results here suggest that it is capable of opening at least 97% of channels in the absence of open-channel block and desensitization. These values are certainly consistent with each other, and they imply that β/α is large (because $p_{o,max} = (\beta/\alpha)/(1+\beta/\alpha)$). However, they are both so close to 100% that there is considerable uncertainty as to exactly how large β/α really is, since changes of affinity and of the channel-opening equilibrium both result in a parallel shift of the concentration–p_o relation under these conditions (see Ogden, Colquhoun & Marshall, 1987). This corresponds, in terms of classical ideas about receptors, to the well-known fact that it is difficult to distinguish between changes in affinity and changes in efficacy for agonists of high efficacy. In principle a more accurate numerical test would be provided by a partial agonist for which the maximum p_o value would provide a more precise estimate of β/α. Our results (Fig. 6) show that ACh itself is a partial agonist at positive membrane potentials, where at saturating concentrations

only 41 % of channels can be opened, i.e. β/α is about 0·7. This low value for β/α results mainly from the greatly increased value of α at positive membrane potentials. However, the very short open times make it difficult to detect brief interruptions of openings at positive membrane potentials and so prevent a test of the multiple-opening hypothesis in this potential range.

The predictions of the burst structure at low concentration made from the data given here differ from those observed by Sine & Steinbach (1986) in BC3H-1 cell line. The main differences in BC3H-1 are the longer gaps due to oscillations between open and shut doubly bound states and an additional component of short gaps which probably represent brief closures to an additional receptor state. These differences are accounted for in their proposed activation scheme by slower rate constants for channel opening and closing, and by an additional closed occupied state beyond the open state. As discussed below, at high concentration, the range of ACh concentrations for activation and the maximum p_o are similar to those found here (Sine & Steinbach, 1987).

The origins of co-operativity

For all the mechanisms considered here, and for any other in which two molecules must be bound before opening can occur, the equilibrium response at low concentrations must be proportional to the square of the free agonist concentration, and the slope (n_H) of the Hill plot must approach 2 at low concentrations. This is true regardless of whether the binding reactions themselves show co-operativity, and whether the binding sites are equivalent or not. Results at the lowest concentrations tested here (5–10 μM) are consistent with this hypothesis (see Fig. 7). Brief openings occurring with a frequency consistent with opening of singly occupied channels have been reported at very low agonist concentrations of suberyldicholine (4–20 nM, Colquhoun & Sakmann, 1985) and these should result in a shallower Hill slope at very low concentrations (see Dionne *et al.* 1978, and Adams, 1981, for evidence on this point). However, p_o could not be measured in that concentration range with the present methods.

The presence of co-operativity, or of non-equivalence, in the binding steps is reflected in the steepness of the p_o curve, e.g. in the rate at which the Hill slope declines towards unity as agonist concentration is increased. The Hill slope at the half-maximum response ($p_o = 0{\cdot}5\, p_{o,max}$) is 1·6 or 1·7 for the results at negative membrane potentials. The results are fitted perfectly well on the assumption that there is no co-operativity or non-equivalence in the binding reaction ($K_1 = K_2 = 77\ \mu$M at -120 mV, $32\ \mu$M at $+100$ mV). They are, however, also consistent with either inherent negative co-operativity in the binding reactions or with non-equivalence of the binding sites, though any substantial degree of negative co-operativity or non-equivalence would require that β/α was very large indeed (otherwise the p_o curve would be too shallow to fit the data). Although consistent with the p_o curve alone, high values of β/α were shown above to predict infrequent and very brief gaps within bursts compared with those observed at low concentrations. If they occurred, such gaps would not be resolved at the present recording resolution and it would become necessary to propose a different explanation, not connected with channel activation by ACh, for the gaps within

bursts that are seen at low ACh concentration. The arguments presented by Colquhoun & Sakmann (1985), in particular the differences of gap frequency and duration observed with different agonists, strongly support the multiple-opening hypothesis for the 20 μs gaps within bursts at low ACh concentration. From eqns (7) and (8) this places upper limits on β and k_{-2} which are not consistent with a high degree of negative co-operativity or non-equivalence of binding site affinities.

The results are also consistent with the possibility that the binding sites are indeed non-equivalent, but the effect of this on the slope of the p_o curve (e.g. on n_H) is cancelled by some inherent positive co-operativity in the binding. It was found that the observations at both negative and positive membrane potentials are consistent with a slight (2-fold) degree of positive co-operativity if this is combined with non-equivalence (to the extent of 10- or 20-fold) in the binding sites.

Dependence on membrane potential

A number of comparisons may be made of data obtained at membrane potentials of -120 and $+100$ mV in these experiments. The changes in the concentration–p_o relation on going from negative to positive membrane potentials can be accounted for almost entirely by two factors; a large decrease in the affinity of ACh for the open ion channel, thus reducing the effects of open-channel block, and by the relatively low β/α, which gives a maximum p_o of 0·41 at positive potentials.

Desensitization. First, the characteristics of desensitization were not noticeably changed. The clusters of openings were of similar duration, as may be seen by comparing values given in Tables 1 and 2. Also, the gaps between clusters were not noticeably different, usually of several tens or hundreds of seconds long at both potential levels. These results suggest that the voltage dependence of the rate of desensitization is small compared for instance with that of the channel open lifetime, although it is not clear how these results compare with the voltage dependence seen in whole-cell currents (Magazanik & Vyskocil, 1970; Scubon-Mulieri & Parsons, 1978; A. B. Cachelin & D. Colquhoun, unpublished observations). In the present case experiments done at potentials close to the equilibrium potential for calcium ions ($+110$ mV; internal calcium concentration assumed to be 200 nM) showed no alleviation of desensitization when compared with those at negative potentials, suggesting that calcium influx is not essential for the occurrence of desensitization, in agreement with experiments in which internal and external calcium are buffered to low levels (A. B. Cachelin & D. Colquhoun, unpublished observations). However, an influence on the extent of equilibrium desensitization would probably not have been detected in these experiments.

Channel closing and opening rates. The analysis of lifetimes at positive membrane potentials posed a number of problems that will be dealt with in detail in another paper. The mean duration of the apparent channel open lifetime was found to be much shorter, about 90 μs, at $+100$ mV than at -120 mV, where the mean value (at low concentration) was 1·5 ms. These values imply an e-fold increase of the shutting rate constant, α, for 78 mV of depolarization. This is similar to the voltage dependence estimated by various kinetic methods at more negative potentials under similar conditions (Magleby & Stevens, 1972; Anderson & Stevens, 1973; Neher & Sakmann, 1975; Adams *et al.* 1977*a*, 1981; Colquhoun & Sakmann, 1985). The results

of curve fitting show that β/α was much reduced at +100 mV, declining from about 32 at −120 mV to 0·7 at +100 mV, an e-fold decrease for about 58 mV depolarization. These results indicate that the channel opening rate constant, β, has only a small dependence on membrane potential, decreasing e-fold for about 230 mV of depolarization. This result supports previous suggestions, from experiments over a narrower range of potentials, that the potential dependence of the channel opening rate is small (Magleby & Stevens, 1972; Neher & Sakmann, 1975; Colquhoun & Sakmann, 1985). The voltage dependence of β, although it is, as expected, in the opposite direction to the voltage dependence of α, is surprisingly small. If the voltage dependence of the channel closing rate is due to movement of charge during the conformation change of channel shutting, as proposed by Magleby & Stevens (1972), one might expect the reverse charge displacement to occur during channel opening and that as a result the rate constant would have a greater potential dependence than that observed here. For this theory to be tenable it is necessary to propose that the energy barrier between shut and open states is asymmetric with respect to the membrane field, being closer (in terms of the fraction of the field) to the shut than the open conformation.

Affinity of ACh binding. The results of curve fitting suggest that there is a slight increase in binding affinity when the membrane is depolarized from −120 to +100 mV. When the two binding constants are assumed equal, there appears to be a 2·4-fold increase of affinity, corresponding to an e-fold increase in about 250 mV of depolarization. This is unexpected because a cationic molecule binding to a site in the membrane electric field would be expected to show a decrease of affinity. The observation that the competitive effect of (+)-tubocurarine showed no dependence on membrane potential (under similar conditions to those used here) suggests that the agonist binding site is outside the electric field (Colquhoun *et al.* 1979) though their measurements were over a smaller range of negative potentials, so a small effect might have been missed. Structural data for the receptor are also consistent with the view that the agonist binding sites are outside the membrane.

If the change of affinity is genuine (it could result from attempts to fit incorrect mechanisms to the data), then the anomalous direction of the change certainly suggests that the agonist binding sites are not in the electric field. It is conceivable that it could result from changes in the conformation of the binding site which originate from potential-dependent conformation changes in distant parts of the molecule that are within the electric field, or from potential-dependent changes in the ionic composition of the solution immediately adjacent to the binding sites.

Comparison with other work

Sakmann *et al.* (1980) made the first observations of desensitization clusters from junctional-type channels in the extrajunctional membrane of denervated frog muscle fibres. The mean cluster duration at 20 μM-ACh of 4 s in their experiments may be compared with the mean value found here of 900 ms. Since the number of channels present in a patch is not known a comparison of the duration of long desensitization gaps is not possible, but qualitatively these appear to be of a similar pattern in the two studies. The mean cluster duration decreases when the ACh concentration is high in both studies, a result consistent with the increased rate of the early component of

desensitization observed in voltage clamp experiments in whole fibres. Indeed, averaging a large number of clusters recorded with 200 μM-ACh in the pipette reproduced the exponential decline of current seen under similar conditions with rapid application of ACh to a voltage-clamped end-plate (A. B. Cachelin & D. Colquhoun, unpublished observations).

Estimates of the open probability as a function of ACh concentration by whole-cell voltage clamp methods are hindered by uncertainty of the number of channels present as well as by desensitization. Estimates of the maximum p_o for ACh have been cited by Adams (1981) as about 0·7 in *Rana esculenta* muscle at 22 °C and −140 mV membrane potential (A. Feltz & P. R. Adams, unpublished data) and about 0·85 in *Rana pipiens* at 10 °C and −80 mV membrane potential (B. Sakmann & P. R. Adams, unpublished data). These suggest a value for β/α of about 8·0. The relation between conductance (as a fraction of the total) and ACh concentration up to 40 μM was similar to that found here. Results at higher concentrations were not reported. Data from single-channel recording in BC3H-1 cell line at low and high ACh concentration have shown that $\beta/(\beta+\alpha)$ is about 0·93 in these cells, although the rate constants themselves are smaller than those at the neuromuscular junction (Sine & Steinbach, 1986, 1987).

Dionne *et al.* (1978) determined the concentration–response relation for carbachol at the frog neuromuscular junction by ionophoresis and reported a maximum response of about 0·5 and positive co-operativity in receptor binding with equilibrium constants of 600 and 60 μM. These results agree fairly well with recent estimates (Marshall & Ogden, 1986; Ogden *et al.* 1987) of carbachol affinity and response maximum made with the methods described here (although it was not found necessary to introduce co-operative binding). However, the low maximum response found by Dionne *et al.* (1978) is most probably due to open-channel block by free carbachol (Ogden & Colquhoun, 1985) suggesting that the $p_{o,max}$ for activation and opening is higher (approximately 0·90) and that carbachol is, like ACh, an efficaceous agonist at nicotinic receptors.

The ACh concentration required to produce 50% receptor activation has been found to be close to 15 μM in extrajunctional regions of frog muscle (Sakmann *et al.* 1980) at the frog end-plate (Dreyer, 1978; Adams, 1981), in rat (Siegelbaum, Trautmann & Koenig, 1984) and human (Adams & Bevan, 1985) primary myotubes and in BC3H-1 cell line (Sine & Steinbach, 1987). Thus, activation appears to occur over the same range of concentrations in skeletal muscle receptor of junctional and extrajunctional types from different vertebrates. Data over a wide range of concentrations have been reported by Takeyasu, Udgaonkar & Hess (1983) for *Electrophorus* receptor reconstituted into lipid vesicles. Their results showed a steep rise of the initial rate of ^{86}Rb uptake when the ACh concentration was increased above 10 μM, with a maximum at about 100 μM and at high concentration an inhibition of the response which was apparent only when the membrane was polarized to −45 mV. At this potential they estimated equilibrium constants of 77 μM for ACh binding to the receptor sites, of 2·5 for the channel opening reaction (β/α in our notation) and 0·8 mM for the inhibition at high concentration, possibly due to open-channel block. These results are similar to those found here, except that the equilibrium for channel opening is about 5-fold smaller, perhaps as a result of

uncertainty about the relation between response and p_0 in their experiments. It will be interesting to compare these parameters for activation of nicotinic receptors by ACh with those from neuronal nicotinic receptors when they become available.

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