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A PATCH-CLAMP STUDY OF THE PARTIAL AGONIST ACTIONS OF TUBOCURARINE ON RAT MYOTUBES

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SUMMARY

1. Single channels activated by (+)-tubocurarine (curare) were recorded from rat myotubes using the patch-clamp technique.

2. The agonist-like action of curare does not result from a contaminant molecule, as the same effects were observed with curare purified by high-performance liquid chromatography.

3. A curare-activated channel can adopt two levels of conductance: full (F) or partial (P). The F state has a slope conductance of 40 pS (identical to that of the acetylcholine (ACh)-activated channel) and the P state has a conductance of 13 pS.

4. At low concentrations of agonist (ACh or curare), the distribution of channel open times is biphasic. The briefer channels may result from the binding of a single agonist molecule whereas the longer-lived channels probably occur following the binding of two agonist molecules.

5. The mean open time of the F state decreases with increasing curare concentration. It is shown that band-width limitations are likely to account for only a very small part of this observed reduction. In contrast, the mean open time of the P state is independent of the concentration of curare. A simple interpretation is that the F state is susceptible to channel blockade by curare, whereas the P state is not.

6. The P state preceded the F state almost as often as it followed the F state; it can also be observed separately from the F state. The fraction of events including a P state increases from about 4% in the presence of 1 μM-curare to 30% at 100 μM-curare. This fraction is also increased by hyperpolarization.

7. When the curare concentration is increased, the F-state frequency first increases and then decreases at higher concentration. This frequency is also decreased by hyperpolarization. The decrease in F-state frequency is probably related to channel blockade by curare; it cannot be wholly accounted for by problems associated with limited time resolution.

8. A synthetic analogue of curare, (+)-tubocurine dimethiodide presents an agonist activity similar to that of curare but with a faster closing rate for both F and P states.

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9. The various actions of curare are summarized in two possible models where the P state is interpreted as either a partially open channel or a channel which is partially blocked.

INTRODUCTION

It has been generally accepted that curare (tubocurarine) inhibits neuromuscular transmission by competing with acetylcholine (ACh) for its binding site on the nicotinic receptor. Thus, as for a typical competitive antagonist, curare causes a parallel shift in the ACh log dose–response curve in first approximation (Jenkinson, 1960). More recently, the primary action of curare in *Aplysia* neurones was reported to be a block of open ion channels (Marty, Neild & Ascher, 1976; Ascher, Marty & Neild, 1978). Similar channel-blocking effects of curare have been since observed at the frog neuromuscular junction (e.g. Manalis, 1977; Katz & Miledi, 1978; Colquhoun, Dreyer & Sheridan, 1979) and in rat ganglion cells (Ascher, Large & Rang, 1979). In addition, it is now clear that curare possesses agonist-like activity. Ziskind & Dennis (1978) first reported that curare depolarized embryonic rat muscles, and subsequent patch-clamp (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) experiments have confirmed the channel-opening ability of curare both in rat myotubes (Trautmann, 1982, 1983; Morris, Jackson, Lecar & Wong, 1982) and in human myotubes (Jackson, Lecar, Askanas & Engel, 1982).

We present here a further study of these paradoxical effects of curare in rat myotubes. A point of special interest concerns the observation of a state of partial conductance for channels activated by curare (see Trautmann, 1982). The molecular interpretation of this state of partial conductance leads to two hypotheses: that it is an intermediate conformation of the channel (i.e. partially open), or that it represents a partial blockade by curare of a normally (i.e. fully) open channel. In examining the concentration dependence of the agonist actions of curare, an attempt has been made to provide a coherent activation scheme that includes consideration of partial, subconductance states, the number of curare molecules required to open the channel and channel block.

METHODS

Culture

Muscles from hind limbs of 18 day old rat embryos were cut into small pieces, kept at 4 °C for 3 h in culture medium, before mechanical dissociation by pipetting. Myoblasts were plated onto gelatin-coated 60 mm plastic culture dishes (Falcon) at a density of 2×10^6 cells per dish and maintained at 37 °C in a humidified atmosphere containing air and 6 % CO_2. The culture medium contained 80 % Eagle's Minimum Essential Medium with glutamine, 10 % Medium 199 with glutamine, 10 % horse serum, 0·05 % penicillin solution (500000 i.u./ml) and 0·5 % streptomycin (20 % solution). All components of the culture medium were obtained from Gibco. The cultures were used following 2–8 days incubation; most exhibited spontaneous contractions, which were first evident about 5 days after plating.

Single-channel recording

The experiments were performed in a bathing solution containing (in mM): NaCl, 150; KCl, 4; $CaCl_2$, 1·5; $MgCl_2$, 1; glucose, 11; HEPES, 5; pH 7·4. Tetrodotoxin (TTX) was added to give a final concentration of 10^{-7} to 10^{-8} M when necessary to arrest spontaneous contractions.

AChCl and (+)-tubocurarine were obtained from Sigma. Stock solutions were stored frozen, and

diluted appropriately for each experiment. Pipette solutions were routinely filtered (0·22 μm; Millipore) and contained ACh (50–200 nM) or curare (1–100 μM) in Ringer solution.

Experiments were performed at room temperature (23–26 °C). The preparation was viewed using Nomarski optics with a total magnification of 256 × (Zeiss). Electrodes were positioned using a hydraulic micromanipulator (Narishige).

Recordings of single-channel currents were performed in giga-seal, cell-attached patches, as has been previously described in detail (Hamill *et al.* 1981). The pipette contained the same solution as that used in the bath, except that glucose was omitted. Care was taken in coating the patch pipettes with a silicone resin (Rhône Poulenc) with an aim to extending the system band width. Both soft (haematocrit) and hard (Pyrex) glass were used to make patch electrodes. Data were FM tape-recorded (Racal 4) with a 0 to 2·5 or 5 kHz band width for later off-line computer (LSI 11/23, DEC) analysis.

Data analysis

The initial step in the data analysis was the digitization at 5–20 kHz of a continuous segment of recording. The data were filtered with an 8-pole, Bessel filter (Frequency Devices) with a cut off (−3 dB point) adjusted to a frequency 3–5 times lower than the sampling frequency. Up to 240000 consecutive points were stored on a floppy disk.

Curare-activated channels containing partial, subconductance states were counted and classed, according to whether the substate appeared before or after the full opening (or alone) as previously described (Trautmann, 1982). Transitions between closed and fully open channel states were continuously monitored and were tabulated by the computer when the signal crossed a variable threshold (usually set about half-maximal amplitude).

After tabulating the channel openings, a correction was made for channels which were undetected due to the limited band width of the recording and analysis systems. This correction factor was calculated as following. In an exponential distribution with time constant τ, the fraction m of events (briefer than a threshold value T; generally 250 μs) which are 'missed' because of limited time resolution is given by:

$$m = \int_0^T \exp(-t/\tau) \Big/ \int_0^\infty \exp(-t/\tau) = 1 - \exp(-T/\tau).$$

Amplitude histograms of channel openings were made in the following way. Blocks of 512 points of digitized data containing one or several channel openings were selected, and each block was transformed into a histogram of amplitudes of all the points in the block, as measured from the centre of the base line of the trace. This type of histogram gives one peak for each current amplitude plus a number of points between the peaks corresponding to the rising and falling edges of the (filtered) currents. As well, the first six bins of the histogram contain events representing the base line, and are well off-scale (see Fig. 3). The data points were fitted with a sum of Gaussian distributions. For each peak (corresponding to one current amplitude), the position of the peak and the value of the standard deviation were determined by eye.

In most of our records, only one channel was open at any given time. In exceptional cases when two agonist-activated channels were open simultaneously, the computer randomly assigned (with a probability of 0·5) the first closing transition to the first or the second opening. The open and closed time distributions of both levels of conductance were calculated. However, for the subconductance state, this analysis was not overly precise because of the low signal to noise ratio and the relatively infrequent occurrence of the substates.

Frequency histograms of both channel closed and open times were constructed and fitted (using least squares) by the computer to a single exponential distribution. When the distribution was biphasic, the computer fitted the slow phase to an exponential, and the additional faster component was fitted by eye using a second computer-generated exponential.

In the analysis of the channel kinetics, openings briefer than about 200 μs were ignored because of the loss of accuracy of our system in measuring events briefer than this duration. On the other hand, very brief closures of the channel, similar to those described for ACh-activated channels (Colquhoun & Sakmann, 1981; Trautmann & Siegelbaum, 1983) were observed, although very rarely. In estimating the mean open time of curare-activated channels, we chose to ignore closures briefer than about 1 ms (see references above). In fact, taking these rare closures into account would not have seriously modified our measurements.

Tests of purity of curare

Curare is not synthesized, but rather is extracted directly from a plant (*Chondodendron fumentosum*). Such a mode of preparation is likely to introduce some impurities into the final product. It was important to examine whether the previously reported paradoxical (agonist-like) effects of curare could be due to such impurities.

As recently described (Trautmann, 1982), we found no channels resembling those activated by curare when the patch pipette contained only Ringer solution. Also, when α-bungarotoxin and curare were present in the pipette, no curare-like channels were observed.

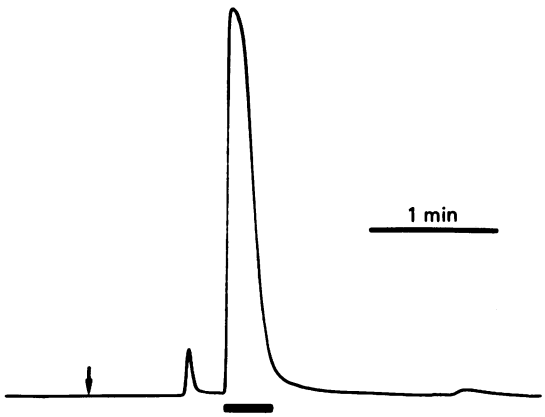


Fig. 1. Elution profile of tubocurarine (Sigma) obtained using high-performance liquid chromatography. The tubocurarine was dissolved in a buffer of ammonium acetate in methanol. The arrow indicates the time of sample injection and the bar corresponds to the period in which the eluted fractions were collected. The vertical scale is in arbitrary units of optical density, measured at 286 nm.

An analysis of the curare (obtained from Sigma) was performed by Drs V. Michon & J. P. Girault, using n.m.r. spectroscopy. First, the compound examined was found to be indeed curare, and secondly, the level of impurities was $< 5\%$, i.e. below the threshold of sensitivity of the method. A further test of the homogeneity of the curare used was provided by a high-performance liquid chromatogram (h.p.l.c.) run by Dr J. P. Henry. Curare was dissolved in a buffer containing 100 mM-ammonium acetate in methanol and run on a h.p.l.c. column. The elution profile, monitored at 286 nm, revealed a major peak and two very minor components (Fig. 1). The major peak was $> 95\%$ of the area above the base line. The eluted fractions corresponding to the major peak (Fig. 1, bar) were collected, lyophilized, and redissolved in physiological solution. The results obtained using the h.p.l.c.-purified curare were not distinguishable from those obtained with the 'crude' curare. This strongly suggests that the observed agonist-like effects of curare on the nicotinic embryonic ACh receptor resulted from curare itself, and not from any impurities which may have been included in the crude product.

Membrane potential of rat myotubes

The resting potential was directly measured in a few cases with high-resistance (classical), intracellular micro-electrodes under current clamp or with patch pipettes in the whole-cell recording configuration (Hamill *et al.* 1981). We found a value of about -60 mV on average. Very similar values were obtained by estimating the resting potential of cell-attached patches using the null potential ($= 0$ mV) found by either interpolation of ACh and curare-activated currents or by extrapolation, assuming a linear $I-V$ relationship.

RESULTS

Curare-activated channels have three conductance states in rat myotubes

As recently reported, channels opened by curare on rat myotubes can adopt essentially two levels of conductance (and rarely a third one, characteristic of the 'adult' channel type; Trautmann, 1982). The modal class of channels has the same

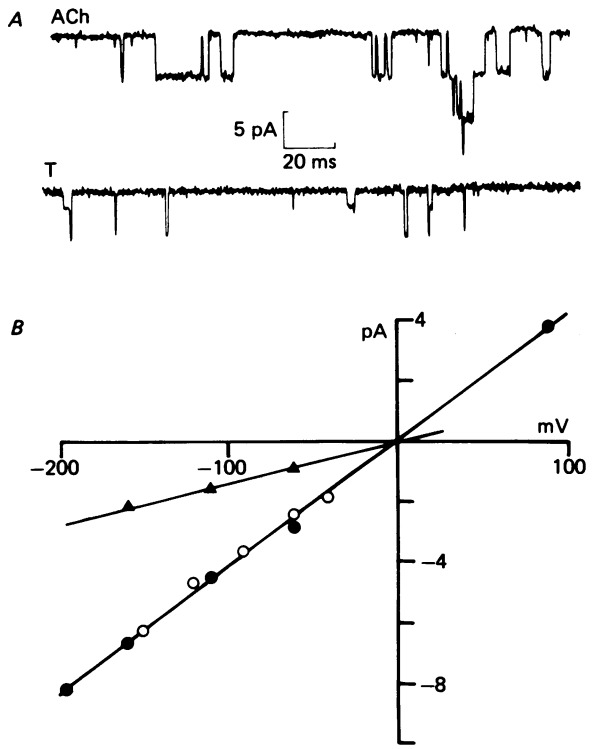


Fig. 2. *A*, single-channel currents recorded on the same myotube, from two cell-attached patches. The pipettes contained 200 nM-ACh in Ringer solution (top trace) and 50 μM-curare (T) in Ringer solution (bottom trace). Patch potential: −150 mV for the ACh trace, and −160 mV for the T trace, with respect to the reversal potential. Data filtered at 2·5 kHz. *B*, *I–V* curves for the currents from the cell shown in *A*. Open circles represent ACh-activated channels, while the filled symbols represent curare-activated channels. Both ACh and curare open channels have a slope conductance of 40 pS; curare alone possesses a second subconductance state (triangles) of 13 pS.

conductance as that of the ACh-activated channels. This is illustrated in Fig. 2, where *I–V* curves were obtained successively from two different, but adjacent patches, not far from one another on the same cell. For the first patch, the pipette solution contained 200 nM-ACh, and the slope conductance of these ACh-activated channels was 40 pS. The second pipette contained 50 μM-curare and channels in this patch had two readily distinguishable amplitudes giving slope conductances of 40 and 13 pS, respectively. The *I–V* relationships were linear over the potential range examined, and the three classes of currents appeared to have a common null (reversal) potential

of about 0 mV. (The resting potential was estimated at the end of the recording by passing from the cell-attached to the whole-cell recording mode.)

Amplitude histograms of the two classes of curare-activated currents were constructed as described in the Methods and are illustrated in Fig. 3. All the digitized points of traces including at least one channel opening were used in making these

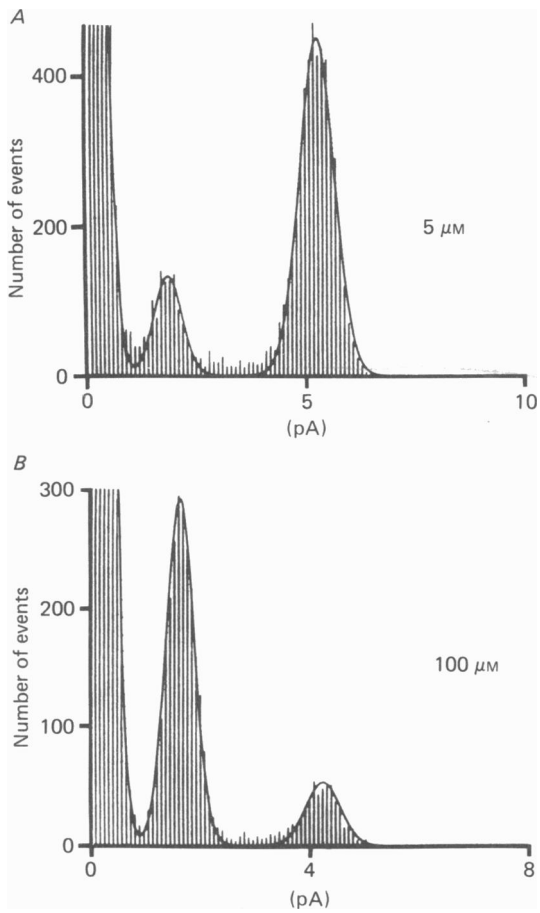


Fig. 3. Amplitude histograms of curare-activated currents. In *A* and *B*, the curve is the sum of three Gaussian distributions. For both cases, the first six bins, corresponding to the points counted in the base line are much larger than indicated (see Methods). Pipette potential, +50 mV; 3 kHz filter; sampling frequency, 10 kHz; events taken into account have durations longer than 0·5 ms. *A*, in the presence of 5 μM-curare, the peaks corresponding to partial (P) and full (F) current levels have amplitudes of 1·9 and 5·3 pA, respectively. The ratio of the integral of the two peaks (P:F) is 1:4. *B*, for a different cell (having a presumably lower resting potential) with 100 μM-curare, the peak amplitudes are 1·6 and 4·2 pA. The ratio of the integral of the two peaks (P:F) is 5:1.

histograms, so that the integral of each peak represents the product $n\tau$ (i.e. n, the number of events times their mean duration, τ). This integral represents the time spent in a given state during the period examined. It is clear that the relative amplitudes of the two current levels described above are independent of the concentration of curare: in the presence of 5 μM-curare, the full conductance state

is 2·8 times larger than the partial one (Fig. 3*A*) while, for another cell in the presence of 100 μM-curare, this ratio becomes 2·6 (Fig. 3*B*). One can observe from the same Figure that in the presence of 5 μM-curare, the time spent in the full conductance state is several times greater than the time spent in the partial conductance state. For 100 μM-curare, this situation is reversed (Fig. 3*B*). A more detailed discussion of this observation is given below.

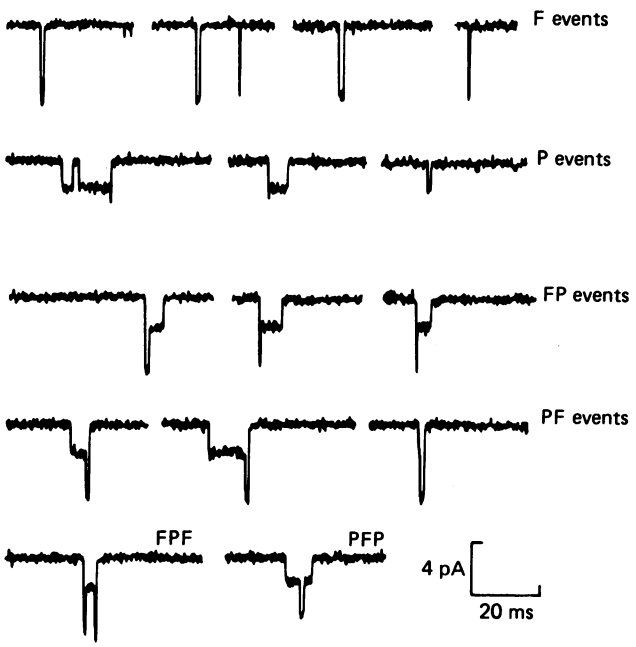


Fig. 4. Various types of single-channel currents recorded in the presence of 100 μM-curare. F events are currents of full amplitude and P events represent a partial subconductance state. Several types of transitions between F and P states were observed (bottom three traces). The pipette potential was +80 mV in all traces except for the PFP event (+50 mV). Data filtered at 5 kHz. For clarity, the currents shown have longer durations than on average.

Many transitions between the partial and full conductance states were observed in the presence of curare (Fig. 2*A*). The probability of occurrence of such transitions was always several orders of magnitude higher than expected for the coincidental collision frequency of two independent openings with conductances of 40 and 13 pS (with no apparent overlap or intermediate closing). Thus, it seems highly probable that a single channel opened by curare can adopt (at least) two different levels of conductance (cf. Hamill & Sakmann, 1981).

As indicated in Fig. 4, the state of *full* conductance has been called the F state, and the *partial* subconductance state, the P state. An 'FP event' then consists of a state of full conductance followed by a transition to the state of partial conductance (with no intermediate closure), and an 'PF event' is the mirror image of an FP event.

Channels displaying a third conductance level (55 pS) with a brief open time were noted very infrequently (Trautmann, 1982). No transition between this level of conductance and the other two was ever observed, suggesting that another type of

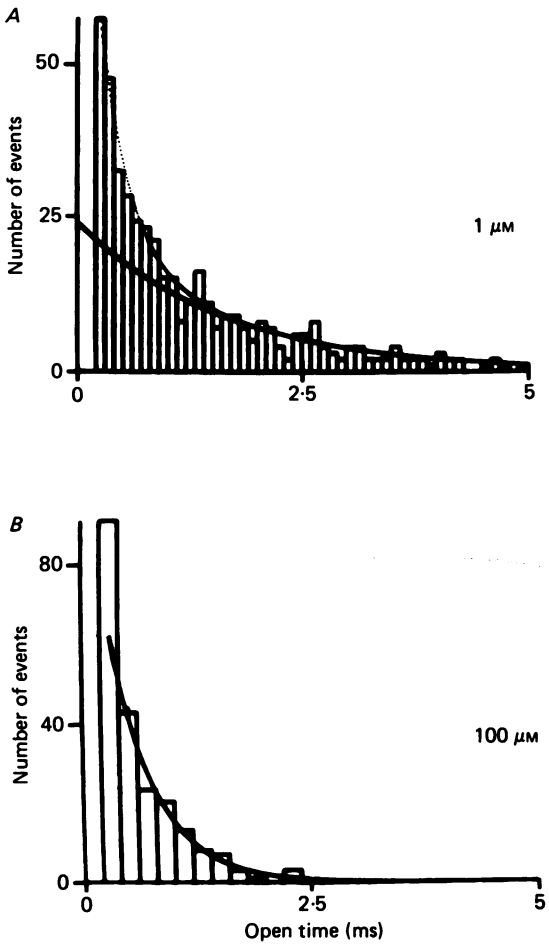


Fig. 5. Distribution of channel open times in the presence of 1 μM-curare (*A*) and 100 μM-curare (*B*). The dotted line in *A* is the sum of two exponentials having time constants 0·3 and 1·7 ms (continuous line). The ratio of the amplitudes of the two components (fast:slow) is 3·6. In *B*, the (single) time constant is 0·5 ms. The histograms were obtained from cell-attached patches on two different, but representative cells, at pipette potentials of +30 mV (*A*) and +50 mV (*B*). Data were sampled at 20 kHz and filtered at 5 kHz; openings briefer than 200 μs were ignored in both cases.

channel was involved. This channel seems likely to be the 'adult type' (Trautmann, 1983), and will not be considered further here.

Distribution of channel open times at low agonist concentration

At low curare concentrations (1–10 μM), the distribution of the open times of the F state is clearly biphasic. This is illustrated in Fig. 5*A*, for a curare concentration of 1 μM. The dotted curve represents the sum of two exponentials, with a fast time constant of 0·3 ms, and slower time constant (continuous line) of 1·7 ms. Such a phenomenon has also been reported by Colquhoun & Sakmann (1981) using ACh at the frog neuromuscular junction. Their hypothesis explaining this observation was that the fast component of the open-time distribution represented ACh receptors

(AChRs) activated by a single ACh molecule, whereas biliganded AChRs would remain open for a longer time. A simple prediction of this model is that the fast component should be more prominent at low agonist concentrations and should disappear (or become much less important) at high agonist concentrations.

Indeed, this prediction seems to apply here to curare-activated channels: as mentioned above, the open-time distribution was clearly biphasic at low curare

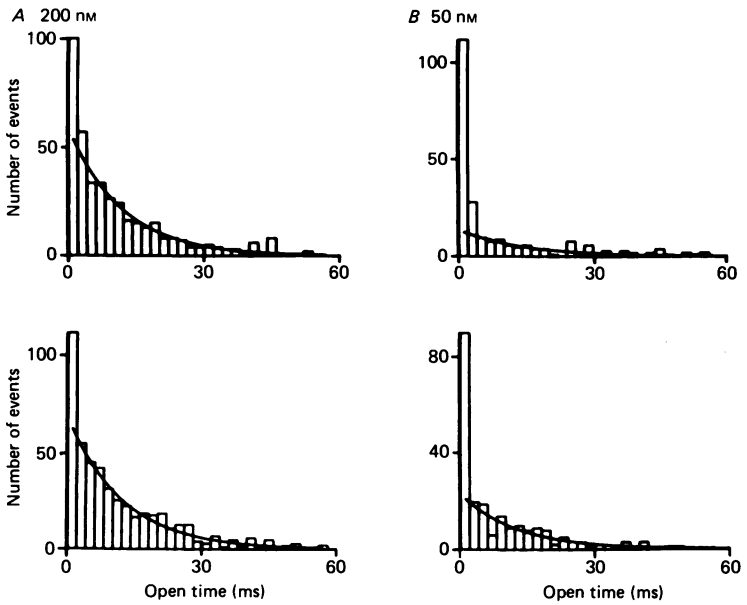


Fig. 6. The effect of ACh concentration on the distribution of channel open times. Four different cells (from the same dish) were examined in the presence of 200 nM-ACh (*A*) and 50 nM-ACh (*B*). Note the greater excess of fast events at low ACh concentration. Time constants (from fits to the slow part of the distributions) are 11·6 and 12·2 ms (*A*, upper and lower), and 14·4 and 12·6 ms (*B*, upper and lower, respectively). Events briefer than 300 μs are not included in the first bin. The pipette potentials were +30 and +20 mV (*A*, upper and lower) and +50 and +100 mV (*B*, upper and lower). Data filtered at 5 kHz.

concentrations (Fig. 5*A*); in contrast, for 100 μM-curare (Fig. 5*B*), the open-time distribution was reasonably well-fitted with a single exponential having a time constant of 0·5 ms. However, the apparent loss of the two-component nature of the open-time distribution at high curare concentration may arise from a speeding up of both fast and slow phases.

In fact, at high curare concentrations, the time constant of the slower component of the open-time distribution is decreased (Fig. 5) and this probably arises from the open-channel blocking activity of curare (see below). Thus, with curare as an agonist, it is difficult to test the hypothesis that the briefest channels observed are associated with receptors activated by a single agonist molecule. It was then of interest to test this idea using ACh as the agonist.

Fig. 6 shows distributions of channel open times obtained from four different cells in the same culture dish, two of them in the presence of very low pipette ACh concentration (50 nM) and two in the presence of an ACh concentration four times greater. It is clear that the fast component of the distributions is more prominent

at 50 nM-ACh than at 200 nM-ACh. Such a difference between low and high ACh concentrations was systematically observed, and is consistent with the idea that channels having very brief open times are activated following the binding of a single ACh molecule (Colquhoun & Sakmann, 1981). Although this population of brief channels is readily distinguishable in patch-clamp experiments, it can be easily estimated that the contribution of these channels to the over-all macroscopic ACh current would be small; as a consequence, the macroscopic current increases with the square of the ACh concentration, i.e. with a Hill coefficient very close to two.

Returning to the populations of very brief channels observed in the presence of low curare concentrations, it is tempting to extrapolate from the results obtained with ACh and to suggest that this population would represent those cholinergic receptors activated by a single curare molecule. The longer-lived curare-activated channels would then have been opened following the binding of two curare molecules. This conclusion may be mildly speculative; at least, it is probably the simplest explanation that can be presently proposed.

The distribution of channel open times for the P state was also examined, in order to see if these partial openings would also have a biphasic distribution of open times (see later). However, the small amplitude of these events results in a low signal to noise ratio, which limits seriously the detection of the briefer openings. In addition, the relative frequency of occurrence of the P state was small, especially at low curare concentrations, as shown below. Thus, the possible presence of a fast component in the P state open-time distribution remains unanswered for the moment.

Effect of curare concentration on channel lifetime

We mentioned above that τ_F, the time constant of the open-time distribution corresponding to the slower F-state channels, was shortened by increasing the concentration of curare (Fig. 5). This observation is presented in a more quantitative manner in Fig. 7. It is apparent that $1/\tau_F$ (○) increased linearly with curare concentration. The slope of the regression line is $2{\cdot}1 \times 10^7$ M^{-1} s^{-1}. Such behaviour is characteristic of a dual action of curare (i.e. a blockade by curare of the channels opened by curare), similar to the dual action of decamethonium described by Adams & Sakmann (1978) at the frog end-plate. The channel-blocking aspect of curare's activity has already been well-documented at the neuromuscular junction of the frog (e.g. Manalis, 1977; Katz & Miledi, 1978; Colquhoun, Dreyer & Sheridan, 1979) and on rat myotubes (Trautmann, 1982).

To our surprise, we observed that, in contrast, the mean open time of channels in the P state, τ_P is practically independent of curare concentration (Fig. 7). If anything, τ_P increases marginally with the curare concentration. In Fig. 7, the interrupted line was drawn with zero slope through the value of $1/\tau_P$ (●) averaged over all curare concentrations used. Thus, a marked difference appears to exist between the F-state and P-state channel: the F state can be blocked while open, while the P-state channel is apparently not susceptible to channel blockade.

It can be calculated that at zero curare concentration, the extrapolated values τ_F and τ_P are 2·09 and 1·58 ms, respectively. The difference between these values may not be significant.

Effect of curare concentration on the probability of occurrence of the different levels of conductance

Reversibility. Transitions were noted between all three conductance levels observed in the presence of curare. If the channel can be either closed (C), partially open (P), or fully open (F), and if these three forms are part of a simple cyclic scheme, a first prediction is that the probability of transitions C → P → F → C should be equal to

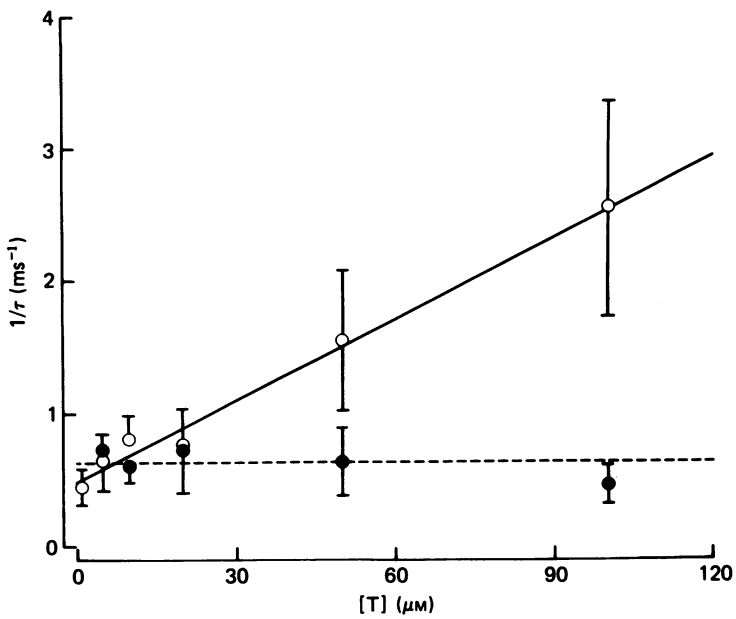


Fig. 7. Effect of curare concentration on the rate of closing of the channel. ○, F events; ●, P events. The least-squares regression line through the open circles has a slope of $2{\cdot}1 \times 10^7$ M^{-1} s^{-1}. At zero curare concentration, $1/\tau_F = 479$ s^{-1}. The dotted line is the horizontal line drawn for a closing rate of the P state averaged over the whole concentration range explored. This closing rate is 631 s^{-1}. Pipette potentials: +30 mV ± 20 mV. The bars give the S.D. for measurements from three to eight cells per point.

the probability of C → F → P → C. Indeed, this prediction of reversibility is not far from being fulfilled: for 365 currents (from twenty-two cells) containing both the sub- and full conductance states, 166 were of the PF type (Fig. 4; i.e. with the substate preceding the fully open state), while 199 were of the FP type (i.e. fully open followed by the substate). This result is in sharp contrast with the observation of Hamill & Sakmann (1981) on rat myotubes: the substate recorded in the presence of ACh at low temperature always followed the fully open state (i.e. a FP event).

Frequency of F events. The effect of curare concentration on the frequency of events including an F state was examined at patch potentials of −90 ± 20 mV. Before averaging data from different patches at each concentration, exceptionally high frequencies were discarded, as they probably correspond to hot spots of high AChR density (see Trautmann, 1982; S. Siegelbaum, A. Trautmann & J. Koenig, in prepara-

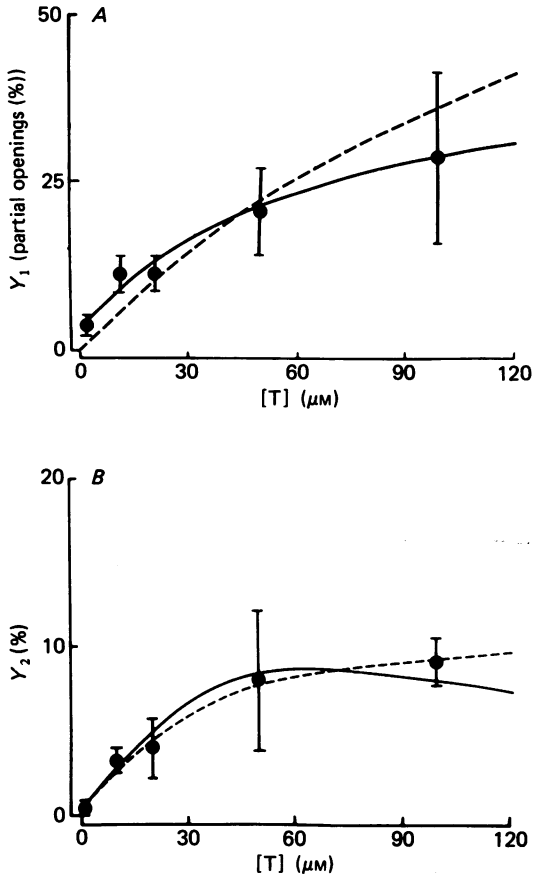


Fig. 8. *A*, Y_1, the percentage of open time spent in the P state as a function of curare concentration. The definition of this time is given in the Appendix for either the 'partially open channel model' (continuous line, eqn. (1)) or the 'partially blocked channel model' (interrupted line, eqn. (2)). *B*, Y_2 represents the ratio of the number of FP events to all F events (i.e. the probability of a full opening being followed by a partial conductance state rather than fully closing). The continuous line is the prediction of the partially open channel model (eqn. (3)) and the interrupted line is the partially blocked channel model prediction (eqn. (4); Appendix). The data were obtained at patch potentials of -90 ± 20 mV. The bars represent the S.D. for measurements from four to six cells per point.

tion). The frequency of channels containing an F event increases between 0 and 10 μM-curare, but then decreases for higher curare concentrations. For example, the frequency of F events was $9{\cdot}1 \pm 3{\cdot}9$ s^{-1} at 10 μM-curare and $1{\cdot}5 \pm 0{\cdot}8$ s^{-1} for 100 μM-curare; thus, a 6-fold reduction. (These values are the average ± S.D. for four to six cells, at an estimated patch potential of -90 ± 20 mV.) At the same time, the mean open time of F events was reduced from 1·25 ms (at 10 μM-curare) to 0·4 ms (for 100 μM). This decrease in channel open time is likely to change *m*, the fraction of events 'missed' because of limited time resolution (see Methods) from 18 to 46%, assuming that all events longer-lived than 0·25 ms are measured. Thus, at most, one would expect about a 30% reduction in the F-event frequency between 10 and

100 μM-curare due to band width limitations, indicating that the large decrease (6-fold) observed is genuine.

Both channel blockade and desensitization could be responsible for the low frequency of channel opening observed at high curare concentration. In contrast to full agonists, no bursting behaviour of the channel was observed at high curare concentration. This situation probably results from a long-lived blocked state of the channels.

TABLE 1. The effect of voltage on the relative frequency of F and P events

Cell	Frequency of F events at *RP*, relative to frequency at *RP*–50 mV	Fraction of events including a P-state at *RP*–50 mV relative to fraction at *RP*	
1	1·6	2·44	
2	2·1	1·50	
3	2·9	1·56	
4	3·3	1·61	
$n = 4$	2·5±0·7	1·78±0·38	(±S.D.)

Curare concentration was 20–50 μM. *RP*, resting potential. n = number of cells.

Fraction of events including a P state. An interesting prediction of a simple cyclic scheme where only three states exist (C, P and F) is that the ratio of the P states over all openings should be independent of the curare concentration and dependent only upon the equilibrium constant for the F⇌P transition. We have already noted from the amplitude histograms presented in Fig. 3, that the fraction of time spent in the P state relative to the time spent in the F state increases at high curare concentrations. In order to test this point further, the fraction of time spent in the P state was calculated, i.e. the number of events in the P state times τ_P divided by the total time spent in either open or blocked state (see Appendix for more details).

One can see in Fig. 8*A* that Y_1, the fraction of time spent in the P state was not constant, but increases markedly with curare concentration. At 1 μM-curare, the P state represented about 4% of the total open time, while at 100 μM-curare, the channel spent about 30% of the time in the P state. This shows that the simple cyclic scheme proposed above is not acceptable. In the Discussion, the possibilities that the P state represents either a partially open channel or a fully open but partially blocked channel will be examined. These interpretations will lead to two, more realistic models. In Fig. 8, the experimental points are fitted with a continuous line for the 'partially open channel model' and with an interrupted line for the 'partially blocked channel model' (see below; Appendix).

Relative occurrence of the FP state. The effect of curare concentration on the probability that a F state is followed by a P state (a FP event) rather than by a direct closure was also investigated. The fraction Y_2, (which is equivalent to the number of FP events divided by the number of F events; see Appendix) is plotted in Fig. 8*B* as a function of curare concentration. It is clear that Y_2 increases with the concentration of curare and that a reasonable fit to the data can be obtained for the predictions of either the partially open channel model (continuous line) or the partially blocked channel model (interrupted line).

Voltage dependence of curare action. The blockade of the cholinergic channel by curare is increased by hyperpolarization. Thus, in the presence of high concentrations

of curare, the mean open time of the channel activated by ACh (e.g. Katz & Miledi, 1978; Colquhoun *et al.* 1979) or by curare (Trautmann, 1982) decreases with hyperpolarization. This classical effect was not further studied here.

The effect of voltage on the frequency of channel opening was examined. For four cells where the signal to noise ratio was good enough at two holding potentials 50 mV apart, the frequency of the F state was 1·6 to 3·3 times higher at the less negative patch

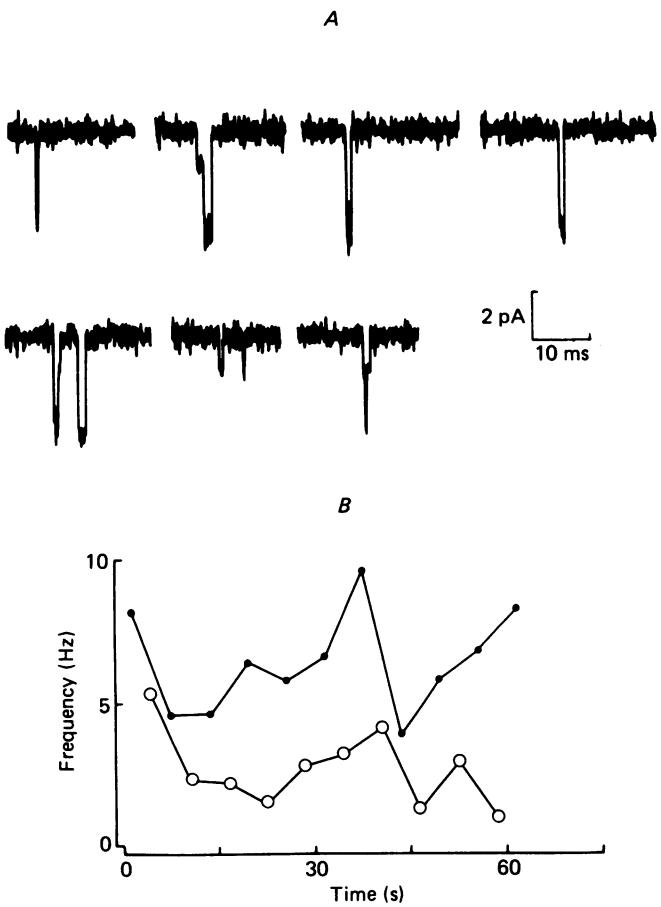


Fig. 9. *A*, single-channel currents recorded in the presence of 100 μM-(+)-tubocurine dimethiodide, a synthetic curare analogue. Holding potential was hyperpolarized 50 mV with respect to the resting potential (*RP*). Filter, 5 kHz. *B*, F-event frequency in the same cell for alternate pipette potentials of 0 (the resting potential, filled symbols) or +80 mV (hyperpolarized, open symbols). The successive command potentials were applied for periods of 3 s.

potential (Table 1). A small part of this effect may arise from problems associated with the detection of briefer events (see Methods). However, band-width limitations cannot account for all of the observed decrease in F-event frequency at hyperpolarized potentials. For example, when τ is reduced from 0·8 to 0·5 ms by a 50 mV hyperpolarization, the fraction of detected events decreases from 73 to 61 %, i.e. a ratio of 1·2, clearly smaller than the ratios of frequencies that were observed. The

decrease in F-event frequency probably reflects the effect of voltage on the fraction of blocked receptors, and hence, on the number of channels able to be activated.

A further effect of voltage was noticed: the frequency of occurrence of the P state relative to the frequency of the F state was increased 1·5 to 2·4 times by a 50 mV hyperpolarization (Table 1). How hyperpolarization could increase the probability of occurrence of a P state will be discussed below.

Channel-opening activity of a synthetic curare analogue

As a further control concerning the possibility that the agonist activity of (+)-tubocurarine may be related to some undetected (but co-purified) contaminant (see Methods), we tested the channel-opening ability of a synthetic curare analogue. The analogue was (+)-tubocurine dimethiodide, kindly supplied by Dr S. Wilkinson (Wellcome Research Laboratories, Beckenham). Single-channel currents recorded in the presence of a pipette concentration of 100 μM of this compound are illustrated in Fig. 9*A*. The characteristics of these channels were very similar to those observed with curare (both 'crude' and purified); in particular, it was possible to observe partial subconductance states. A preliminary analysis suggests that the frequency of events containing a P state is lower. Another difference was that the open time of both F and P events was briefer (data not shown). Additionally, as for curare, we noted a clear voltage dependence of the channel-opening probability. In Fig. 9*B*, the F-event frequency is plotted for successive imposed pipette voltages (lasting about 3 s) of 0 mV (i.e. the resting potential; ●) or +80 mV (hyperpolarization of the patch; ○) in the same cell. One can observe a several-fold reduction in the F-state frequency at the more hyperpolarized potential.

Curare does not open channels in chick myotubes

It was of interest to examine the channel-opening properties of curare in chick myotubes, as it has been reported previously that curare activates AChRs on human myotubes (Jackson *et al.* 1982). We did not observe any curare-activated currents in chick myotubes. It was clear however, that functional AChRs were present as ACh-activated channels could be recorded from the same cells that had failed to respond to curare. Similar results have been obtained by G. D. Fischbach (and colleagues; personal communication).

DISCUSSION

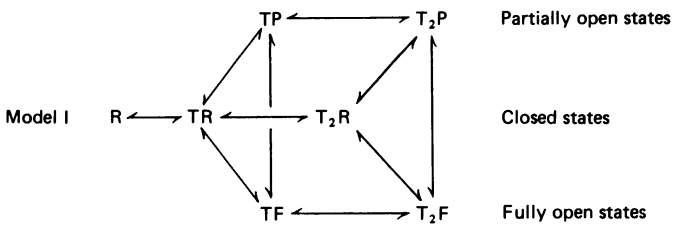
In this paper, a quantitative analysis of some aspects of the agonist properties of curare at the cholinergic receptor of rat myotubes has been attempted. It was suggested that one curare molecule is probably able to cause a brief opening of the channel, a second curare molecule tends to maintain the channel open for a longer time and that a third curare molecule can completely block the open channel. The possible activation of the cholinergic receptor by either one or two curare molecules was proposed as an explanation for the presence of two components with different time constants in the F state open-time distributions. Also, by analogy, the observation that the frequency of brief channels, relative to that of longer-lived channels is greater in the presence of a low ACh concentration than in the presence

of a higher ACh concentration (Fig. 6) supports this hypothesis. A state of partial conductance, similar to the one observed only at low temperature in the presence of ACh (Hamill & Sakmann, 1981) was much more frequently seen here in the presence of curare and at room temperature. This subconductance state can be accessed directly from either the closed state or from the already fully open channel.

Furthermore, the P state is not susceptible to channel block and its relative frequency of occurrence (compared to that of the F state) increases with the concentration of curare and with membrane hyperpolarization. We have raised the question as to whether the P state corresponds to a partially open channel, or to a channel which is fully open but partially blocked. We will show that both possibilities exist, although there is a certain advantage of simplicity in the model where the P state is a partially blocked state.

Model I: the P state is partially open

Let us suppose first that the channel can adopt three conformations: closed, partially open and fully open. It has been suggested above that the F state can be associated with the binding of either one or two curare molecules. On the other hand, for the P state, it was shown earlier that this possibility was not easily distinguishable; nevertheless it is quite conceivable that the P state may also be associated with the binding of either one or two curare molecules. Such a model of activation is described below. As before, F is the fully open state, P is the partial conductance state, R represents closed receptor states and T stands for tubocurarine.



In this scheme, the two isomerisation constants for the F⇔P transitions are:

$$L_1 = [\mathrm{TF}]/[\mathrm{TP}] \quad \text{and} \quad L_2 = [\mathrm{T_2F}]/[\mathrm{T_2P}].$$

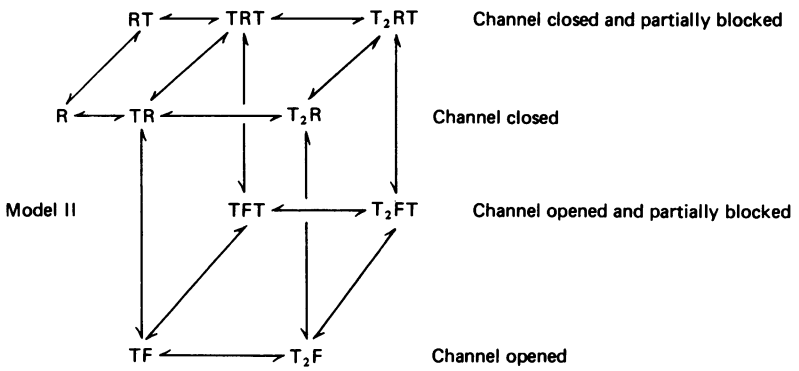
One can easily show (see Appendix) that Y_1, the ratio (time spent in the P states)/(time spent in either open or blocked state) is close to $1/(1+L_1)$ at low curare concentration and shifts towards $1/(1+L_2)$ at high curare concentration. The data illustrated in Fig. 8*A* show that the fraction of small (P) events increases with the concentration of curare; with appropriate values of L_1 and L_2, the partially open channel model (continuous line, Fig. 8*A*) provides a reasonably good fit to the results.

In this model, the channel would presumably have a different conformation in the P state compared to the F state. If the P state corresponds to a partially constricted (open) channel conformation, the narrowing of the channel should occur at a level more *external* than the binding site for classical channel block. This assertion follows directly from the observation that the F state mean open time decreases with increasing curare concentration, whereas the P state open time is unchanged (i.e. the P state is not susceptible to channel block as is the F state, because the blocking site

is inaccessible once the channel has assumed a P conformation). We have not tested this hypothetical asymmetry of channel block in cell-free patches. It thus remains to be seen whether or not a channel-blocking action exists for curare when present on the cytoplasmic side and whether this action would be differential for the F and P states.

Model II: the P state is partially blocked

Let us make an alternative supposition: the channel can adopt only two conformations, open and closed, but in addition it can be either fully blocked (see below) or partially blocked, by one extra curare molecule in both cases. The blocking molecules would bind to two different sites in the channel for the two cases. The site for partial block would have to be accessible with the channel in its closed conformation in order to explain why the P state is not necessarily preceded by a F state. This scheme can be written as below, where R indicates a closed state, F an open state, RT a closed-channel state with a bound molecule of curare, and FT an open, but partially (curare-) blocked state. As previously, we suppose that the receptor can be activated by either one or two molecules of curare.



In such a model, it is easy to see why the relative frequency of occurrence of the P state increases with the concentration of curare: more curare molecules are bound to the receptor in the P states (TFT and T_2FT) than in the F states (TF and T_2F). But, in contrast, at low curare concentration, Y_1, the ratio (time spent in the P state)/(time spent in either open or blocked state) should tend towards zero. This prediction was not perfectly satisfied experimentally (interrupted line, Fig. 8*A*); nevertheless, the partially blocked channel model also gives a reasonably good fit to the data.

In model II, as the site of partial channel block is accessible in the closed conformation, it should be more external than the classical site of full channel block. (The latter, located deep in the membrane (e.g. Colquhoun *et al.* 1979), is accessible to channel blockers only when the channel is open.) Therefore, it is not surprising that a curare molecule bound to the external site can block access to the other, deeper site. This would explain simply why the P state cannot be further blocked by curare.

If the site of partial channel block is not too external, it would also feel part of the membrane potential. Therefore, the occurrence of the P state (relative to that of the F state) should increase with hyperpolarization, as was indeed found

experimentally (Table 1). It thus turns the scales slightly in favour of this model, since an effect of hyperpolarization on a channel block is somewhat more straightforward than an effect of hyperpolarization on a conformational change.

The models proposed above describe the activation of the cholinergic receptor by curare and two possible explanations for the P state; a full description of the action of curare must also include its blocking activity.

Channel block by curare

In this paper (and previously, Trautmann, 1982), we have accumulated several lines of evidence for a channel block of the F state by curare.

(1) τ_F decreases when the curare concentration is increased because the probability of the channel being blocked (added to the probability of closing normally) increases the chances of leaving the open state.

(2) τ_F decreases with hyperpolarization at high curare concentration, indicating that the rate of blocking of the channel tends to be increased with hyperpolarization. (The curare molecule being charged, tends to be 'sucked' into the channel by the membrane field.)

(3) The bursting behaviour of channels activated by a high ACh concentration is abolished in presence of curare, which indicates a slow rate of dissociation of curare from the blocked channel (Trautmann, 1982).

(4) The frequency of channel opening is reduced when the concentration of curare [T] is increased, probably because the fraction of blocked channels depends (in part) on $[T]^3$, whereas the fraction of open channels depends (in part) on $[T]^2$. However, one cannot exclude the possibility that part of the reduction in the frequency of channel openings observed at high curare concentration may result from a curare-induced desensitization.

(5) The frequency of channel opening by both curare and a curare analogue is decreased by hyperpolarization (Table 1; Fig. 9*B*). It is unlikely that the binding of curare is reduced by hyperpolarization. Therefore, it must be that the fraction of receptors able to be activated is reduced by hyperpolarization, perhaps because of an increase in the number of blocked channels. Both on and off blocking rate constants would be implicated here: the effect observed is larger than can be accounted for by the decrease in τ_F described above (point 2), which involved only the on rate constant of channel blockade.

Thus both models should be completed by the branch:

$$\ldots T_2F \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} T_2F_BT,$$

where F_B is the blocked channel. From the data of Fig. 7, it can be calculated that $k_3 = 2{\cdot}1 \times 10^7\ M^{-1}\ s^{-1}$, a value similar to that previously reported for the blocking action of curare (e.g. Colquhoun *et al.* 1979).

Generality of the observed phenomena

It is clear that curare, in binding to the classical ACh receptor site not only provokes competitive antagonism of agonist binding, but may also act as an agonist itself, resulting in the opening of the channel. As well, curare binds to at least one other site on the receptor/ionophore complex, giving rise to its channel-blocking activity.

Several other molecules probably share this property of curare in binding to these two classes of sites: decamethonium (Adams & Sakmann, 1978), procaine (Israël & Meunier, 1979) and various cholinergic agonists (Sine & Steinbach, 1983). This raises the interesting possibility that the two sites possess certain homologies. Recent work on the sequencing of the four polypeptide subunits of the cholinergic receptor (of *Torpedo*) by the group led by Numa (e.g. Noda, Takahashi, Tanabe, Toyosato, Kikyotani, Furatani, Hirose, Takashima, Inayama, Miyata & Numa, 1983) reveals important structural homologies and lends support to this hypothesis (see also Conti-Tronconi, Gotti, Hunkapiller & Raftery, 1982).

The picture which emerges from these facts is subtle rather than clear-cut. All cholinergic ligands may have at least four modes of action: competitive antagonism, activation (i.e. channel opening), channel block (of the 'fully' open channel) and desensitization. Our observations concerning the partial conductance state suggest a fifth mode of action (partial activation or partial block). Simple quantitative differences in the various rate constants could result in one predominant mode of action for any particular condition. For example, one may consider: (1) concentration of agonist: at equilibrium, a low concentration of ligand can induce a marked desensitization without a large activation (e.g. Feltz & Trautmann, 1982), since the affinity of the desensitized receptor is higher than that of the resting receptor (for review, see Changeux, 1981); (2) voltage: at least for the frog neuromuscular junction, channel blockade by curare is an important phenomenon only at fairly hyperpolarized membrane potentials (Manalis, 1977; Katz & Miledi, 1978; Colquhoun *et al.* 1979; Shaker, Eldefrawi, Aguayo, Warnick, Albuquerque & Eldefrawi, 1982); (3) differentiation: the agonist activity of curare is much more marked for rat embryonic ACh receptors than for adult receptors (Ziskind & Dennis, 1978; Trautmann, 1983); (4) species: curare possess agonist activity on rat and human myotubes, but not on chick myotubes. This point may be related to the previous one: as judged from channel kinetics, the cholinergic receptor of chick myotubes resembles more closely the adult type (Schuetze, 1980), in contrast to the situation in the rat (Sakmann & Brenner, 1978; Fischbach & Schuetze, 1980); (5) agonist dependence of channel kinetics: curare opens channels with a longer mean open time, than does a curare analogue, but with a briefer open time compared to ACh. Similarly, at frog end-plate, the mean lifetime of cholinergic channels depends on the agonist type (e.g. Katz & Miledi, 1973).

A final point concerns the number of binding sites contained within the channel. Here, we propose that there might exist two distinct sites that result in partial or full channel blockade by curare. It would be somewhat surprising if there was a single blocking site within the ACh-activated channel, given the diversity of blocking ions and drugs previously reported. As well, the relationship between these blocking sites and those that bind permeant cations remains to be resolved.

APPENDIX

We give here briefly the equations used to obtain the various curare-dependent parameters given in the Results for the two models proposed above. As presented in the Discussion, model I is the partially open channel model and model II is for the fully open, partially blocked channel.

Estimation of Y_1

Y_1 is defined as the fraction of time an open channel spends in the P state (relative to the time spent in either open or blocked state). The experimental points (see Fig. 8*A*) were obtained by counting the different types of events in the following way, where n_P is the number of events including a P state, isolated or associated with a F state (a PFP event would be counted twice). n_F is defined similarly. The ratio n_F/n_P is multiplied by d_P/d_F, which corrects for undetected events (d_P and d_F are the percentages of F and P events which are detected, i.e. longer-lived than a threshold value of 250 μs). τ_P is the mean open time of a P state and τ_F^0 is the mean open time of F states at zero curare concentration. One then may calculate:

$$Y_1 = \frac{1}{1+\frac{n_F \tau_F^0}{n_P \tau_P}.\frac{d_P}{d_F}}.$$

Model I. The continuous curve of Fig. 8*A* was calculated with eqn. (1) derived from model I, where

$$Y_1 = \frac{1}{1+\frac{[TF]+[T_2F]}{[TP]+[T_2P]}},$$

i.e.
$$Y_1 = \frac{1+[T]/K_P}{1+L_1+(1+L_2)[T]/K_P}. \tag{1}$$

K_P is the dissociation constant of curare from T_2P.

One observes that when $[T] \to 0$, $1/Y_1 \to 1+L_1$,
and when $[T] \to \infty$, $1/Y_1 \to 1+L_2$.

In Fig. 8*A*, $L_1 = 24$, $L_2 = 0{\cdot}6$ and $K_P = 1{\cdot}2$ μM.

Model II. The interrupted line of Fig. 8*A* was calculated with eqn. (2) derived from model II, where

$$Y_1 = \frac{1}{1+\frac{[TF]+[T_2F]}{[TFT]+[T_2FT]}},$$

$$Y_1 = \frac{1}{1+K_2/[T]}. \tag{2}$$

K_2 is the dissociation constant of curare from its channel site (responsible for the partial blockade). In Fig. 8*A*, $K_2 = 175$ μM.

Estimation of Y_2

Y_2 is defined as the probability that a F state is followed by a P state rather than simply closing. Experimentally, Y_2 is the ratio of the number of FP events divided by the number of F events observed in the same experiment.

Model I. According to model I, one should have

$$Y_2 = \frac{l_1[\mathrm{TF}] + l_2[\mathrm{T_2F}]}{\alpha_1[\mathrm{TF}] + [\mathrm{T_2F}](\alpha_2 + [\mathrm{T}]k_3)}.$$

k_3 has been defined above and the other rate constants are as follows:

Thus,

$$Y_2 = \frac{l_1 + l_2[\mathrm{T}]/K_\mathrm{F}}{\alpha_1 + (\alpha_2 + [\mathrm{T}]k_3)[T]/K_\mathrm{F}}, \tag{3}$$

where K_F is the dissociation constant of curare from $\mathrm{T_2F}$. This equation was used to draw the continuous line in Fig. 8*B*, with $K_\mathrm{F} = 30\ \mu\mathrm{M}$, $l_1 = 12\ \mathrm{s}^{-1}$, $l_2 = 250\ \mathrm{s}^{-1}$, $\alpha_1 = 3000\ \mathrm{s}^{-1}$ and $\alpha_2 = 230\ \mathrm{s}^{-1}$. Note that $\alpha_2 + l_2 = 1/\tau_\mathrm{F}^0$ and that the rate of closing of the briefest channels in the F state $= \alpha_1 + l_1$.

Model II. According to the partially blocked channel model, one should have

$$Y_2 = \frac{[\mathrm{T}]k_2([\mathrm{T_2F}] + [\mathrm{TF}])}{[\mathrm{TF}]\alpha_1 + [\mathrm{T_2F}](\alpha_2 + [\mathrm{T}]k_3)},$$

and (4)

$$Y_2 = \frac{k_2([\mathrm{T}] + K_\mathrm{F})}{\alpha_1 K_\mathrm{F}/[\mathrm{T}] + \alpha_2 + [\mathrm{T}]k_3},$$

where α_1 and α_2 have the same meaning as that in model I, and k_2 is the rate of binding of curare to the site responsible for partial blockade. This equation was used to draw the interrupted line in Fig. 8*B*, with $\alpha_1 = 3000\ \mathrm{s}^{-1}$, $\alpha_2 = 480\ \mathrm{s}^{-1}$ (i.e. $1/\tau_\mathrm{F}^0$) and $k_2 = 2{\cdot}2 \times 10^6\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$. ($K_\mathrm{F}$ and k_3 were estimated above.)

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