J. Physiol. (1978), **277**, *pp.* 153–176
With 13 *text-figures*
Printed in Great Britain

LOCAL ANAESTHETICS TRANSIENTLY BLOCK CURRENTS THROUGH SINGLE ACETYLCHOLINE-RECEPTOR CHANNELS

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(*Received 16 August 1977*)

SUMMARY

1. Single channel currents through acetylcholine receptor channels (ACh channels) were recorded at chronically denervated frog muscle extrajunctional membranes in the absence and presence of the lidocaine derivatives QX-222 and QX-314.

2. The current wave forms due to the opening and closing of single ACh channels (activated by suberyldicholine) normally are square pulses. These single pulses appear to be chopped into bursts of much shorter pulses, when the drug QX-222 is present in addition to the agonist.

3. The mean duration of the bursts is comparable to or longer than the normal channel open time, and increases with increasing drug concentration.

4. The duration of the short pulses within a burst decreases with increasing drug concentration.

5. It is concluded that drug molecules reversibly block open end-plate channels and that the flickering within a burst represents this fast, repeatedly occurring reaction.

6. The voltage dependence of the reaction rates involved, suggested that the site of the blocking reaction is in the centre of the membrane, probably inside the ionic channel.

INTRODUCTION

The binding of cholinergic agonists to the ACh receptor results in an increase in membrane conductance. It has recently been shown that each open channel contributes a square pulse of conductance: the open channel has a constant conductance and the transitions from closed to open and back take a very short time (Neher & Sakmann, 1976). The exponential decays of normal end-plate currents thus reflect the distribution of open-times for receptors activated by the brief pulse of nerve-released ACh (Magleby & Stevens, 1972).

It is well known that many drugs can alter the decay kinetics of end-plate current. In particular, some drugs cause the appearance of end-plate currents which decay as the sum of two exponentials. The local anaesthetics and especially two lidocaine derivatives (QX-222 and QX-314) have been extensively studied (Steinbach, 1968*a*, *b*; Maeno, Edwards & Hashimura, 1971; Kordaš, 1970; Beam, 1976*a*, *b*; Katz & Miledi, 1975; Ruff, 177; Adams, 1976, 1977).

Ruff (1977) discussed several alternative models for the local anaesthetic action

and fitted most of his data to a specific sequential reaction scheme in which a closed channel can open, and an open channel can either close or reversibly bind drug. The open channel, with a drug molecule bound, would have a much lower conductance than a free open channel; it would be practically blocked. Such a sequential blocking mechanism seems particularly attractive since it has been postulated to hold for a number of similar phenomena and may be a general feature of gated channels (e.g. Adams, 1976, 1977; Armstrong, 1966, 1971; Hille, 1977*b*; Strichartz 1973; Marty, Neild & Asher, 1976).

In this study we examine the effects of QX-222 and QX-314 on the currents through individual channels in the extrajunctional regions of denervated frog skeletal muscle fibres. We test our results against the predictions of the sequential blocking model mentioned above, and find excellent agreement. Numerical analysis yields the individual rate constants of the blocking reaction, from which inferences can be made about the location of the local anaesthetic binding site.

METHODS

Recording and preparation

Details of the method of recording single channel currents in frog muscle membrane are given in a separate publication (Neher, Sakmann & Steinbach, 1978). In short, single fibres of 4–6 weeks denervated frog (*Rana pipiens*) muscles were voltage clamped with a conventional two microelectrode arrangement. In addition an extracellular semi-micropipette (1–5 μm i.d.) was placed onto the surface of the fibre in order to isolate electrically a small patch of muscle surface. The interior of this pipette contained Ringer solution and the cholinergic agonist suberyldicholine (sub) at a concentration of 0·1 μM. It was connected to a virtual ground circuit, which held its potential to ground and at the same time measured the current flowing through the pipette (band width approx. 500 Hz). This current, under favourable conditions, is identical to the voltage clamp current traversing the patch of membrane under study. A primary requirement for this equivalence, and also for obtaining an adequate signal to noise ratio in the patch recording, is a good seal between glass pipette and muscle surface. For this purpose, the muscle fibres were enzyme treated (Betz & Sakmann, 1973) in order to expose the muscle membrane. After denervation a gradient of ACh receptors develops along the fibre with highest density at the location of the former end-plate, and almost negligible density far away from it. For any given fibre a location was found at which, under the given experimental conditions, on the average less than one channel was open at a time. Current recordings were stored on analogue tape and replayed for analysis.

The local anaesthetic was added to both the solution in the external pipette and to the bath Ringer solution. In some preliminary experiments the drug was added to the pipette solution only, with similar results. QX-222 and QX-314 were gifts from Bertil Takmann, Astra Pharmaceuticals to A. B. Steinbach. Ringer solution had the following composition (mM): 117 NaCl, 1·8 $CaCl_2$, 2·5 KCl, 2 phosphate buffer, adjusted to a pH of 7·4–7·5. Tetrodotoxin was usually added to the bath at a concentration of approx. 10^{-7} M. The preparation was cooled by means of two Peltier coolers to temperatures between 6 and 10 °C. Individual kinetic parameters were normalized to a temperature of 8 °C, assuming a Q_{10} of 3 (Beam, 1976*a*).

Data analysis

Before the addition of drug single channels have a square wave appearance (Figs. 1, 2). In the presence of drug, bursts of pulses appear with the individual pulse length much shorter than the square wave. However, the duration of the burst is comparable to, or longer than the normal square wave. The beginning and end of a burst will be termed channel opening and closing, and the transitions within a burst will be termed channel blocking and unblocking.

Two types of analysis were performed. In a first method all available data were displayed sequentially on a storage oscilloscope, burst lengths were measured directly from the screen,

and averages calculated. Two types of problems arose with this kind of analysis. The first one resulted from limited time resolution, i.e. channels shorter than a certain minimum open time could not be unambiguously recognized as such. To avoid this problem, only channels longer than a predetermined threshold length were counted. This over-estimates the mean channel open time. Therefore, the threshold value was subtracted from the measured mean value. The correction is quantitatively correct for the case of an exponential distribution of open times.

The second difficulty resulted from the fact that occasionally channel contributions overlap. It turned out that the most practical way to account for this was to treat overlapping events as if they were single openings and to correct the mean for the resulting overestimate. The correction applied was

$$\bar{t} = \bar{t}'.(1+\tfrac{1}{2}\bar{p}_o)^{-1}, \quad (1)$$

where $\bar{p}_o$ is the probability that any number of channels (1, 2 or more) are open (or in their bursting states) at a given time, $\bar{t}$ is the corrected mean value and $\bar{t}'$ is the measured mean value. This correction holds as a first order approximation if the probability distribution for one, two or several channels, being open simultaneously follows a Poisson law. This assumption appears to be valid for these experiments (Neher *et al.* 1978). Data were discarded if the applied correction was larger than 20 %.

In a second analysis on a subset of data from experiments with the best signal to noise ratio, the recordings were digitized at sampling intervals between 0·5 and 2 msec. The digitized records were inspected on a computer generated display, and times of transitions between states (e.g. channels switching on, being blocked or unblocked, etc.) were marked by manual input into the computer. The computer recorded time, polarity (upward or downward jump), and for certain calculations, also mean current values of preselected segments before and after the jumps. The set of these values represented a complete (idealized) description of the switching and blocking behaviour of the channels. Switching kinetics were characterized by three types of histograms, derived from these data:

(*a*) *open duration histograms*: histograms of time intervals between a downward step (channel opening or unblocking) and the next following upwards step (channel closing or blockade);

(*b*) *block duration histograms*: histograms of intervals between an upward step (channel closing or blockade) and the next following downwards step (channel opening or unblocking);

(*c*) *Pulse correlation functions*: histograms of intervals between downward steps and any following downward steps.

Histograms of these three categories usually had the shape of an exponential or an exponential plus a constant. Such curves were fitted to the experimental data and curve parameters were determined by an iterative least square procedure. Goodness of fit tests (χ-square test) were performed. For the χ-square tests it was assumed that individual values distributed Poisson-like with the mean value given by the theoretical curve.

The two analysis problems mentioned above were accounted for in the following way during this second set of analyses. The limited band width of the recording apparatus showed up as a deficiency in the first one or two bins of the histograms (the very shortest opening or blocking events could not be recognized as such). For this reason the first one or two points of the histograms were usually excluded from the exponential fitting routine. The problem of overlap of several channels was avoided by selecting data segments with low enough density of events (overlap probability $< 0{\cdot}05$).

RESULTS

Single channel recordings in the absence of local anaesthetics. Square pulse current wave forms could be observed in a patch electrode recording, if a patch of membrane with an appropriate channel density was selected, and if the pipette contained agonist in the concentration range 10^{-7}–10^{-6} molar. Fig. 2*A* shows such a recording for 0·1 μM-suberyldicholine (sub) as an agonist, at a holding potential of -150 mV. Amplitudes of the most pronounced square pulses were consistent with a channel conductance of 25–35 pS. Usually smaller channels occurred as well, the smaller size of which, however, has been shown to be an artifact (Neher *et al.* 1978). (The measured

conductance of these channels was smaller because they were located under the pipette wall).

In the absence of local anaesthetics, channel open times were distributed exponentially, as expected for a Poisson process. The mean open time (or the inverse of the decay constant for an exponential fitted to the histogram) depended exponentially on membrane potential (Fig. 7), again as expected. The pulse correlation function (defined in Methods) was usually flat (Fig. 13), indicating that channels opened in a statistically independent fashion.

The action of QX-222. In the presence of QX-222 single channels no longer appeared as simple square waves, instead bursts of shorter pulses were seen (see Fig. 1). With

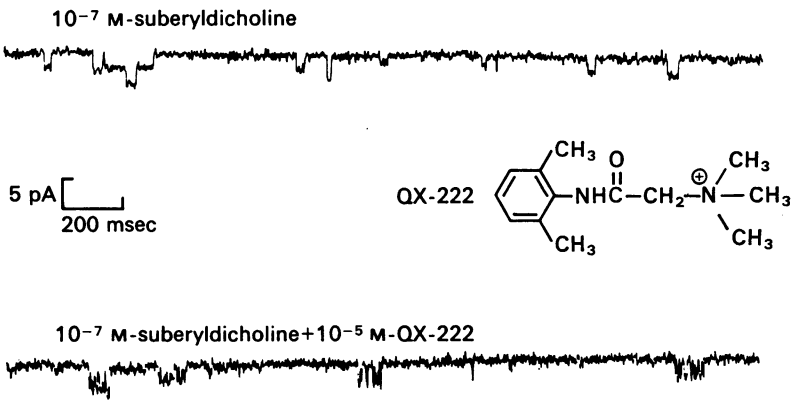

Fig. 1. Single channel recordings in the absence and presence of 10 μM-QX-222 at -120 mV holding potential and 6·5 °C. Inward current (conductance increase) is plotted downwards. In the absence of local anaesthetic, single channels contribute square pulse-like wave forms; in its presence they cause bursts of much shorter pulses. The insert gives the structural formula of QX-222.

very little local anaesthetic in the bath (between 1 and 5 μM) single channels basically maintained their shape, but showed short upward (closing) spikes during their open period (Fig. 2*B*). With more QX, the frequency of these 'blocking' events increased (Fig. 2*C*). Finally, at very high concentrations ($\approx$ 50 μM) the current contribution of a single channel was more like a sequence of irregularly spaced downward (opening) needles (Fig. 2*D*). The described phenomena were voltage dependent in the sense that membrane hyperpolarization increased the frequency of blocking events, increased the mean time of the block, and lengthened the duration of the pulse bursts. An example of a single, exceptionally long burst at better time resolution is given in Fig. 3.

A simple molecular mechanism of drug action. The phenomenology outlined above indicates that channels can undergo a transition from their open state into another state with small or zero conductance. It indicates that for QX-222 this state usually is short lived and the channel rapidly returns to the open state. In addition, the dependence on QX-concentration depicted in the sequence of Fig. 2*A–D* points towards a bimolecular reaction involving the receptor-channel and the QX molecule.

These are the essential features of the sequential blocking scheme put forward by Adams (1976, 1977) and Ruff (1977).

$$\begin{array}{c} (nT + R \rightleftharpoons T_nR) \\ \alpha \Big\| \Big\downarrow \beta \\ T_nR^* + Q \underset{F}{\overset{G}{\rightleftharpoons}} T_nR^*Q. \end{array} \qquad (2)$$

The first step in this scheme is a simplified version of the channel opening reaction (R represents receptor, T transmitter, while an asterisk denotes channels in their open conformation) leaving the question open how many agonist molecules are required to open a channel. Some states and transitions are enclosed in brackets in scheme 2 because we cannot distinguish between state R and T_nR in the present experiments. The second part of the scheme is the local anaesthetic binding (Q stands for QX-222), which is considered to result in a low conductance 'blocked' state T_nR^*Q. For the remainder of the Results section, the data will be described in terms of this intuitively simple scheme, while in the Discussion the data will be considered in a more general fashion and other schemes examined.

Fig. 4 shows an idealized current trace, in which the correspondence between steps in the reaction scheme and events in the current recording is pointed out (see Figure legend). It also indicates three quantities (t_o: open time, t_b: block time, t_g: duration of pulse groups) which can be measured in such a recording. Measuring a fourth quantity, the interval between the appearance of pulse groups, does not provide any useful information unless the number of channels in the patch of membrane under study is known. As is apparent from the records of Figs. 1 and 2, the three quantities t_o, t_b and t_g are random variables. Scheme (2) allows predictions to be made about probability density functions and mean values of these variables. For instance, one of the measurable quantities is the frequency of occurrence of pulses with lengths between t and $t + \Delta t$ (as a function of t), or in other words, the probability $p_o(t)$ that a channel closes (or undergoes blocking) between time t and $t + \Delta t$ under the conditions that it was open at time zero, and that it did not close (or get blocked) transiently in between. As a first step towards this let us first consider the quantity $P_o(t)$ which gives the probability that the channel is still open at time t under the condition that it had been open at time zero and that it did not close (or get blocked) transiently in between. The rate of change of this quantity is proportional to the sum of the reaction rates leading away from the open state:

$$\frac{\mathrm{d}}{\mathrm{d}t} P_o(t) = -P_o(t) \sum_{j \neq o} k_{o,j}, \qquad (3)$$

where $k_{o,j}$ are the rate constants of the reaction scheme (2) which lead away from the open state (the available states considered are o: open, b: block, c: closed).

Note that eqn. (3) is a special form of the Master equation in which all the off-diagonal elements of the corresponding system of linear equations are crossed out. The Master equation approach (for an application to end-plate channel switching see Stevens, 1972) treats probabilities P_{ij} of finding a system in state j at time t under the condition that it had been in state i at time zero, irrespective of what happens in between. Therefore all possible transitions leading towards and leading away from state j have to be considered. When analysing a single channel recording, an additional restriction can be set up: the system does not change its state during the time interval in question. This simplifies the treatment immensely by uncoupling the differential equations.

The solution to the differential equation can be given immediately:

$$P_0(t) = \exp\left(-\sum_{j\neq 0} k_{0,j}t\right). \tag{4}$$

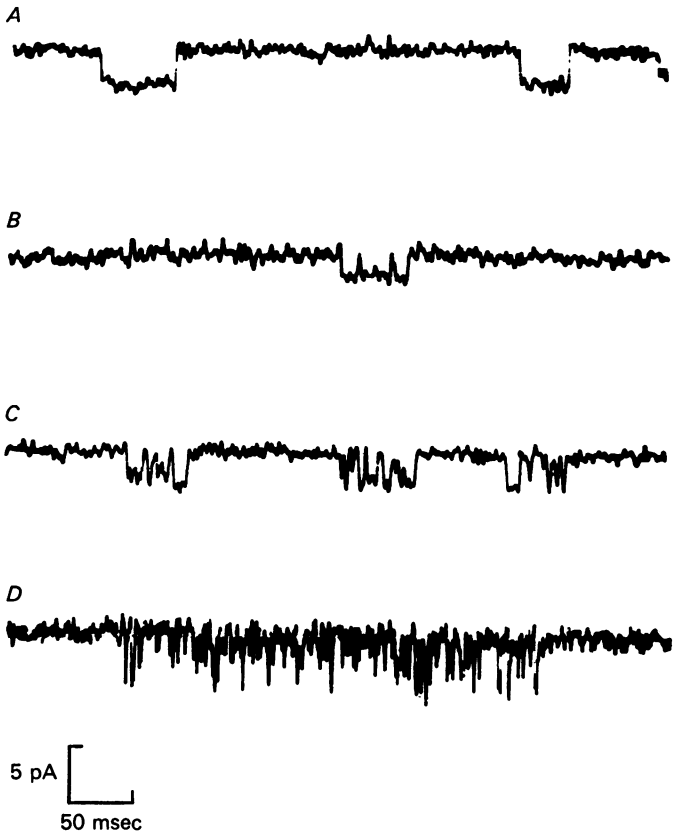

Fig. 2. Concentration dependence of QX-222 action. *A*, two single channel openings in the absence of local anaesthetic (0·1 μM-suberyldicholine present). *B*, 0·1 μM-sub + 5 μM-QX-222: note that two short blocking events occur during the open period of the single channel. *C*, 0·1 μM-sub + 10 μM-QX; blocking events are more frequent, such that the channels spend almost half of their time in the blocked state. *D*, 0·1 μM-sub + 50 μM-QX; a single exceptionally long duration channel. Blocking events are so frequent that the individual short open times can no longer be resolved; the flickering now appears as a series of downwards (opening) needles. Note that the spacing in between needles is about the same as the block intervals in *C*. Holding potential: −150 mV in parts *A* and *D*, −120 mV in parts *B* and *C*. Temperature: 7 °C in parts *A*, *B*, *C*, 9 °C in part *D*.

The quantity $p_0(t)$ which we are actually looking for is proportional to the derivative of $P_0(t)$:

$$p_0(t) \propto \exp\left(-\sum_{j\neq 0} k_{0,j}t\right). \tag{5}$$

Using the rate coefficients of scheme (1)

$$p_0(t) \propto \exp\left(-[\alpha(V)+G(V).c_Q]t\right), \tag{6}$$

where c_Q is the concentration of the local anaesthetic drug, and all the rate constants are functions of voltage. Thus, the mean time $\bar{t}_o$ of an open channel is

$$\bar{t}_o = [\alpha(V)+G(V).c_Q]^{-1}. \quad (7)$$

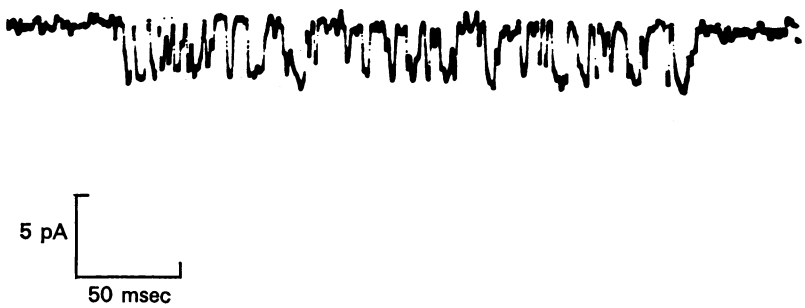

Fig. 3. A single exceptionally long-lasting burst at better time resolution. Note that the transitions between conducting and non-conducting states are *not* necessarily faithfully represented in this trace; that is to say, the time course of the actual transition of the channel from one state to another is not shown in the Figure. The time of these molecular transitions is distorted both by the time constant of the apparatus and the baseline noise. Detailed studies at the highest possible time resolution have indicated that the observed transition time for channels is determined by the time constant of our apparatus and all we can say is that these transitions are too fast to be observed in our experiment (Neher *et al.* 1978). Recording in 10 μM-QX-222, 6 °C, −150 mV.

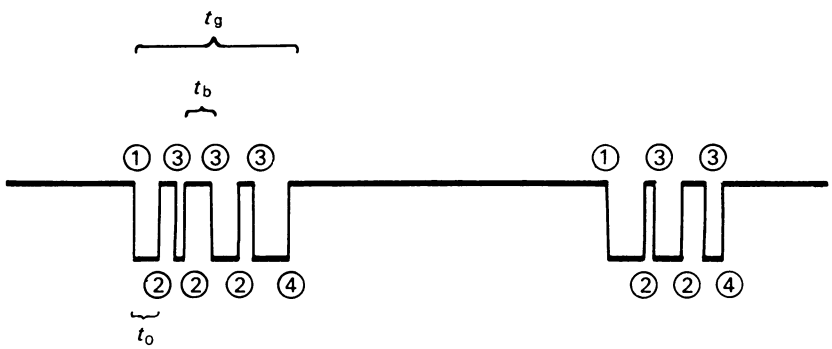

Fig. 4. Idealized current trace. Points marked by ① refer to channel openings, which are represented by $T_nR \rightarrow T_nR^*$ in reaction scheme (2), ② marks blocking reactions: $T_nR^* \rightarrow T_nR^*Q$, ③ marks unblocking $T_nR^*Q \rightarrow T_nR^* + Q$ and ④ marks final closing $T_nR^* \rightarrow T_nR$. The time intervals corresponding to the variables t_o (open time) t_b (block time), and t_g (duration of pulse groups) are indicated.

An analogous consideration of the blocked time intervals leads to

$$\bar{t}_b = [F(V)]^{-1} \quad (8)$$

since the unblocking reaction (with rate constant F) is the only reaction pathway leading away from the blocked state in scheme (2).

This simple treatment requires that blocked periods can be distinguished from intervals between bursts. As will be discussed later, this is possible in the case of QX-222, where traces with very low channel densities were analysed, such that interburst intervals are very long. If these long intervals are counted anyway in the process of histograms forming (see Methods section), then the distribution of blocked

plus closed times will be the sum of two exponentials. However, for all the QX-222 analyses the second component was practically zero.

Calculating the distribution of group- (or burst-) durations, t_g, brings in an additional problem: termination of a burst is not simply leaving a single state, but leaving the set of states $T_n R^* + T_n R^* Q$ (see scheme (2)). The corresponding reaction rate is

$$\alpha(V).P(o|o \text{ or } b),$$

where $P(o|o \text{ or } b)$ is the conditional probability that the channel is open, given it is in one of the two states open or blocked. Under the simplifying assumption that the blocking – unblocking equilibrium is relatively fast with respect to the closing reaction

$$P(o|o \text{ or } b) \approx \frac{F(V)}{F(V)+G(V).c_Q}$$

and so

$$\bar{t}_g = [\alpha(V)]^{-1}.\left(1+\frac{G(V).c_Q}{F(V)}\right). \tag{9}$$

To sum up the predictions of the reaction scheme (2): (1) The measurable quantities t_o (open time), t_b (blocked time) and t_g (groups or burst time) are random variables with exponential distributions. (2) The mean open time decreases with increasing drug concentration according to eqn. (7). (3) The mean group- (or burst-) duration increases with increasing drug concentration according to eqn. (9). (4) The mean blocked time is independent of drug concentration.

Note that the mean group duration (eqn. 9) is completely specified without any further adjustable parameters, once the mean blocked time and the mean open time have been determined. Its measurement is thus an independent test for the validity of the model.

The lengthening of burst duration with increasing drug concentration is a consequence of the fact that the model does not allow a direct transition from the blocked to the closed state. Eqn. (9) thus is a specific test for the assumption that the blocked channel cannot undergo the transition to its closed conformation. Another consequence of the assumption that the blocked channel cannot close is the prediction that the sum of all the short open periods, on the average, is equal to the mean open time of the unmodified channel. This feature is implied in eqn. (9).

Voltage- and concentration dependence of QX-222 action. For a detailed analysis of channel switching under the influence of QX-222 a great number of durations for t_o and t_b (open time and block time, see Fig. 4) were measured and histograms formed, as described in the Methods section. Two of these histograms are shown in Fig. 5 for the case of a fibre in 0·1 μM-sub plus 10 μM-QX-222 at -120 mV, 6 °C. The mean open time (Fig. 5*A*) was relatively long under these conditions. The mean open time was determined in two fashions: it was estimated as 5·2 msec as the time constant of the exponential least square fit (τ_o, see Fig. 5). Alternatively, direct calculation of the mean (with a correction for limited time resolution, see Methods section) gave $\bar{t}_o$ of 4·4. The goodness of fit test (χ^2-test) showed that the distribution could very well be approximated by an exponential. The mean block time (Fig. 5*B*) was very short and close to the limit of time resolution in this case (such limited time resolution shows up as a deficiency in the first few points of the histograms). Nevertheless an estimate could be obtained from the tail of the distribution, assuming that it is an

exponential. This gives the values $\tau_b = 2{\cdot}3$ msec and $\bar{t}_b = 2{\cdot}4$ msec. Data segments with low activity and non-overlapping bursts were selected to simplify the interpretation as explained above.

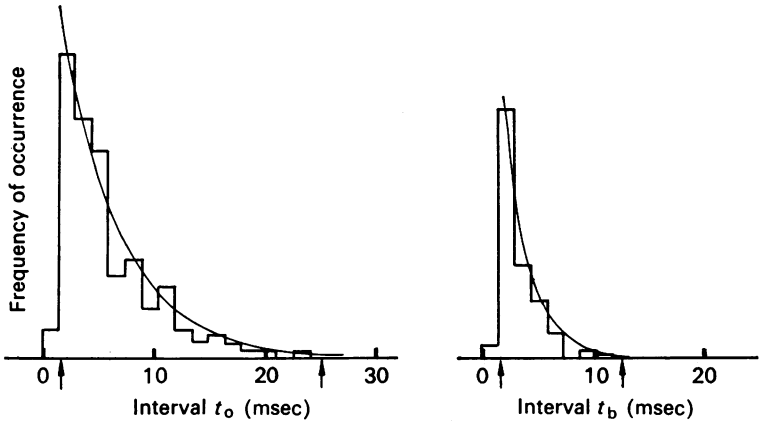

Fig. 5. Histograms of time intervals for t_o (part *A*) and t_b (part *B*) (for definitions of t_o and t_b see Fig. 4). Recordings from a cell in 0·1 μM-sub and 10 μM-QX-222 at -120 mV, 6 °C, were analysed. Exponentials were fitted to the section in between arrows by a least-squares routine. Goodness of fit test gave a reduced χ^2 of 0·8 (46 degrees of freedom) for part *A* and of 1·37 (21 degrees of freedom) for part *B*. 215 events were analysed in each case, using 1·5 msec long bins for the data.

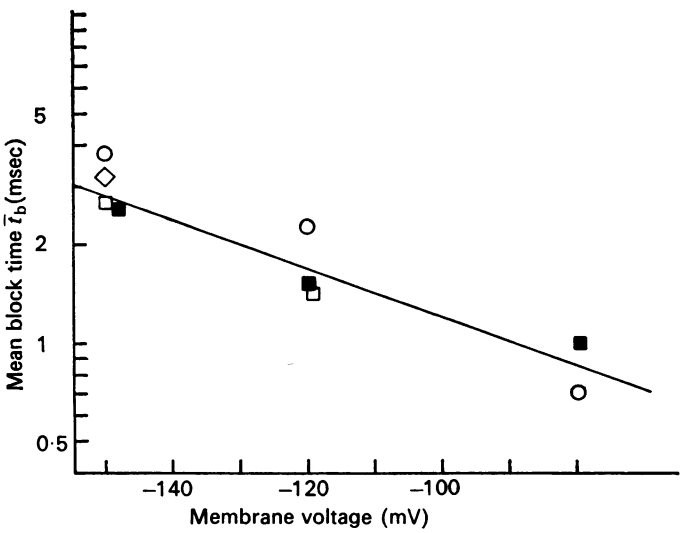

Fig. 6. Mean block time, $\bar{t}_b$, versus membrane voltage. All values in this and the following Figures are normalized to a temperature of 8 °C, assuming a Q_{10} of 3. —○—, 5 μM-QX-222; —□—, 10 μM; —■—, 20 μM, —◇—, 50 μM (the 50 μM-value was estimated from an oscilloscope recording like Fig. 2*D*, counting the number of spikes per time interval). The continuous line is a least-squares fit to the data.

This kind of analysis was performed on three experiments (seven different fibres) under a variety of conditions, and the results were compared against the predictions of the model. It turned out that all of the measured quantities are voltage dependent,

that $\bar{t}_b$ is independent of QX concentration, and that $\bar{t}_o$ decreases with increasing concentration of QX-222 in the way as predicted by the model. The mean block time, $\bar{t}_b$, is plotted against voltage in Fig. 6. It is seen that, for each voltage, values scatter around some mean, but there is no obvious trend with respect to concentration. The voltage dependence of F, the backward rate constant of the blocking reaction, was therefore determined as a linear regression of all the experimental values in a log plot (see eqn. (8)).

$$F(V) = [\bar{t}_b(V)]^{-1} = 4{\cdot}5 \times 10^3 \exp(0{\cdot}0168 \times V), \tag{10}$$

where V is in mV and $F(V)$ is in sec^{-1}.

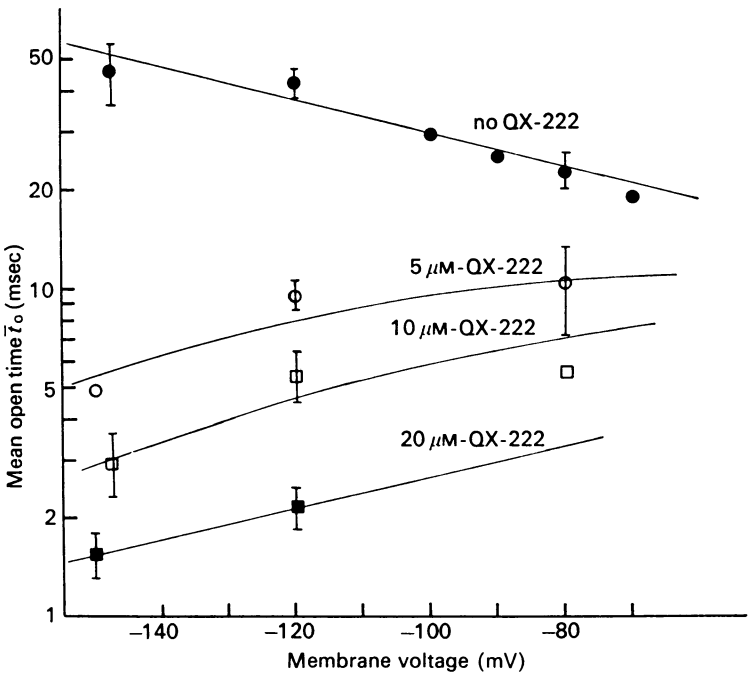

Fig. 7. Mean open time, $\bar{t}_o$, *vs.* membrane voltage for 0·1 μM-sub and different concentrations of QX-222. In the absence of QX, —●— (uppermost curve), $\bar{t}_o$ is the normal mean channel open time. In the presence of 5 μM-QX-222 (—○—), 10 μM-QX-222, (—□—) or 20 μM-QX-222 (—■—) it is the mean duration of the short pulses within a burst. Continuous lines are best fits of eqn. (8) to the data, assuming exponential dependence of $G(V)$ on voltage. Error bars are ± S.E. of the means.

Mean open times $\bar{t}_o$ were found to depend both on concentration of QX-222 and on voltage. Voltage dependence will be considered first: $\bar{t}_o$ is plotted against voltage for different concentrations of QX-222 in Fig. 7. The solid lines are theoretical curves according to eqn. (7), where $\alpha(V)$ has been derived directly from a linear regression in this log plot using the $c_Q = 0$ data. We obtained the relationship

$$\alpha(V) = 112.\exp(0{\cdot}012.V), \tag{11}$$

where α is in sec^{-1} and V is in mV (giving an e-fold change in α for an 83 mV change in V). This relationship is shown as the top line in Fig. 7. The $\bar{t}_o$ data in the presence of QX-222 were analysed in terms of eqn. (7) to obtain $G(V)c_Q$. Individual fits

(continuous lines in Fig. 7) showed that $G(V)$ depended exponentially on voltage with e-fold changes per 60 mV for the case of 5 μM and 10 μM-QX-222, and an e-fold change per 83 mV for the case of 20 μM-QX. Thus, the forward rate of blocking is a quite strongly voltage-dependent process.

The differences between the voltage dependencies of the three cases may not be significant, considering the fact that there are only -120 and -150 mV values available for the case of 20 μM.

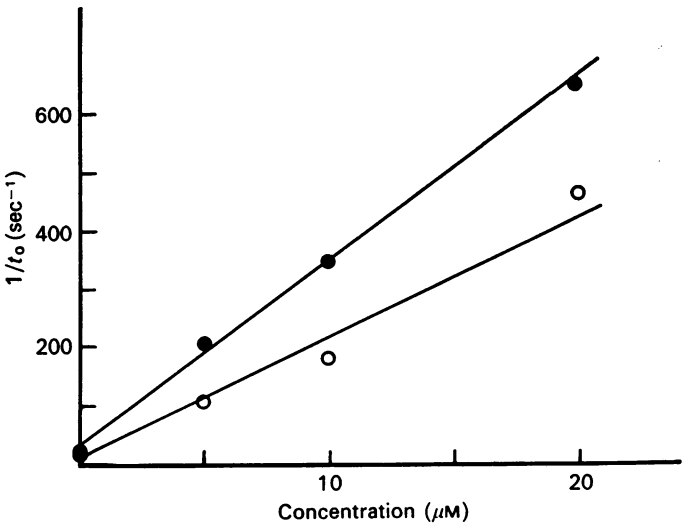

Fig. 8. The inverse of the open time ($\bar{t}_o^{-1}$) versus concentration of QX-222 at -120 mV (—○—) and -150 mV (—●—). The continuous lines are linear regressions using the weights 4, 2, 2, 1 (from left to right) for the individual points to account for differing standard errors. Note that these lines have non-zero intercepts on the ordinate, since these intercepts equal $\alpha(V)$.

The forward rate constant, $G(V)$, can be obtained from a plot of $\bar{t}_o^{-1}$ against concentration. This plot should have a slope of $G(V)$ and an intercept of $\alpha(V)$. Such plots for membrane potentials of -120 and -150 mV are shown in Fig. 8. The linearity of the plots indicates that the blocking is a bimolecular reaction. From the slopes we found the following values for $G(V)$: $2{\cdot}05 \times 10^7$ sec^{-1} M^{-1} at -120 mV and $3{\cdot}12 \times 10^7$ sec^{-1} M^{-1} at -150 mV. This, again, corresponds to a voltage dependence of the forward rate, which changes e-fold per 71 mV. Assuming an exponential dependence of G on V, we obtained the relationship

$$G(V) = 3{\cdot}8.10^6 \exp(-0{\cdot}014.V), \tag{12}$$

where G is in sec^{-1} M^{-1} and V is in mV. The equilibrium dissociation constant for the binding reaction, which according to scheme (2) is given by $F(V)/G(V)$ is therefore

$$K_D = \frac{F}{G} = 1{\cdot}2.10^{-3} \exp(0{\cdot}031.V), \tag{13}$$

where K_D is given in M. At -120 mV a value close to 30 μM is obtained.

The durations of the bursts or pulse groups were comparable to or longer than the mean channel open times in the absence of QX-222. As shown in the previous section,

the mean burst length as a function of concentration can be predicted from theory when α, F and G are known. The predictions are compared against the experimentally observed burst lengths in Fig. 9. Data points are plotted for zero QX-222 and 50 μM-QX-222, the two most extreme cases under study. Continuous lines are calculated according to eqns. (9), (10), (11) and (12). Note that at -120 mV, QX-222 lengthened the bursts 2·3-fold with respect to the unmodified channel open time. This ratio is predicted by the theory within experimental resolution. Small deviations at the most hyperpolarized value may be explained by an overestimate of the voltage dependence in the derived relationships for the rate constants (eqns. (10), (11) and (12)).

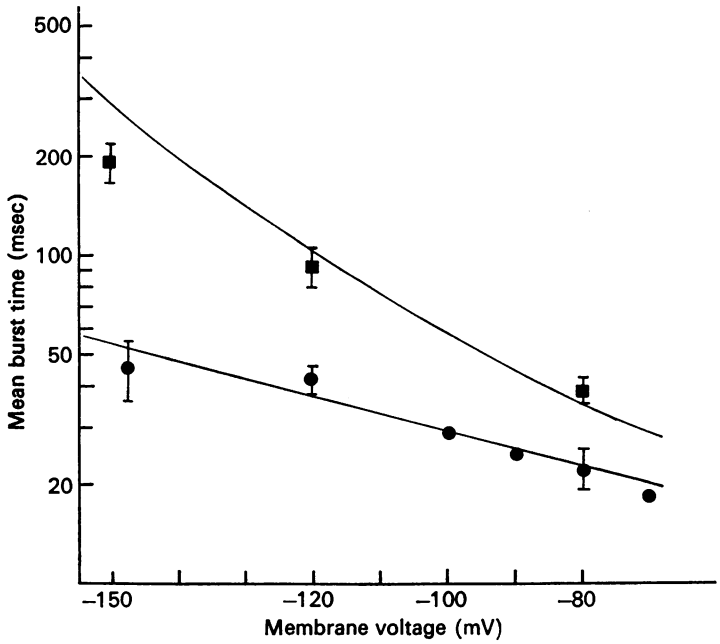

Fig. 9. Mean burst duration versus membrane voltage. The mean burst duration in the presence of 50 μM-QX-222. (—■—) is compared to the mean channel open time in the absence of QX-222 (—●—). The line fit to the data for 50 μM-QX-222 was calculated from eqn. (9) using no free parameters, as described in the text. A perfect fit can be obtained if a less voltage dependent QX-dissociation constant (e.g. $K = F/G = 4{\cdot}2 \times 10^{-4}$ exp $(-0{\cdot}021V)$ is used.

Direct comparison was difficult for cases with lower QX-concentrations since the lengthening with respect to unmodified channel open time was small and was masked by cell to cell variations in the absolute value of α. However, the typical burst lengthening showed up in each case when comparing values from individual fibres at different potentials. In the absence of QX, mean channel open time increased 2·3-fold when hyperpolarizing from -80 to -150 mV. In the presence of QX this ratio was further increased due to the strong voltage dependence of the term G/F in eqn. (9). Table 1 gives a comparison between experimental and predicted ratios.

The relatively close agreement between experiment and theory shows that a blocked channel cannot undergo the conformational change into its closed state;

that is, there is no direct reaction pathway from the blocked to the closed state. Deviations from the predicted curve might be interpreted as evidence for the existence of such a pathway, but if so, the reaction rate from blocked to closed could be at most of the order of $\alpha/10$.

TABLE 1. Ratios of mean channel open time (or burst lengths) at -150 and -80 mV

Concentration of QX-222 (μM)	0	5	10	20	50
ratio $\bar{t}_{150}/\bar{t}_{80}$, experimental	2·14	3·0	4·2	5	6·5
ratio $\bar{t}_{150}/\bar{t}_{80}$, theor. eqn.	2·3	3·2	3·9	5·3	8·4

The conductance of the blocked and open states. There are two questions related to the conductance values of channels under QX-222. (1) Is the conductance of the open state modified by the presence of QX? (2) Is the conductance of the blocked state measurably different from zero?

Concerning the first question, mere inspection of the data indicates that there cannot be much influence of QX-222 on the conductance of the open state (see Fig. 2). A more quantitative comparison is difficult, since there was appreciable variation in channel conductance between different fibres and only rarely could experiments be done with the same fibre and different solutions. In one such instance, an amplitude analysis was performed both before QX action and in the presence of 10 μM-QX-222. Before QX action, conductance of the most prominent steps was 28 ± 2 pS (mean $\pm$ S.E. of mean); in the presence of QX-222 it was $29{\cdot}8 \pm 0{\cdot}6$ pS. Thus, there is no significant difference.

To answer the second question we tried to establish an upper limit for the conductance of the blocked state. We analysed the available -150 mV data, which, due to the voltage dependence of $\bar{t}_b$, have the longest block times. Blocked intervals were looked for which lasted between 7 and 14 msec, then mean current values for 10 consecutive data points (5 msec) were calculated and compared with base line values both before and after the corresponding burst. It was found that the blocked sections were shifted with respect to the base line by $0{\cdot}004 \pm 0{\cdot}04$ pA (mean $\pm$ S.E. of mean) at a mean conductance of the open state of 3·8 pA. Thus, there was no measurable current contribution of the blocked state and we may safely say that any such contribution was smaller than 5 % of the open state current.

Two possible errors must be taken into account, when considering these numbers.

1. There might have been unblocking events during the blocked periods under study which were not recognized as such, being too short. A rough estimate for the contribution of this error can be obtained by assuming that an unblocking event is overlooked if it is shorter than the sampling interval (the time constant of the recording apparatus was comparable to or shorter than the sample interval).

The average number N_{miss} of such overlooked openings per observation period is

$$N_{miss} \approx \frac{t_{obs}}{\bar{t}_b} \cdot \frac{t_{samp}}{\bar{t}_o},$$

where t_{obs} is the observation period (5 msec) and t_{samp} is the sample interval. Putting in the numbers for -150 mV from Fig. 6, a value of 0·35 is obtained. The contribution of these spikes is small, however, since their mean duration is $t_{samp}/2$. Therefore

$$\Delta G = 0{\cdot}35G \frac{t_{samp}}{2t_{obs}} \simeq 0{\cdot}02G,$$

where G is the conductance of the open state.

2. The other possible error comes from the fact that short intervals between bursts may be mistaken for blocked periods. For an estimate of that error, the rate of transition from the open state to the blocked state has to be compared with the rate of transition from the open state to the closed state. In both cases the transition rates have to be multiplied by factors which allow for the fact that we do not consider all possible transitions but only those resulting in blocked (or closed) periods between 7 and 14 msec length.

With worst case values (mean interval between bursts assumed to be 500 msec, lowest concentration of QX) a maximum error of about 2% (related to the measured value for the blocked channel conductance not to the open channel conductance) is obtained.

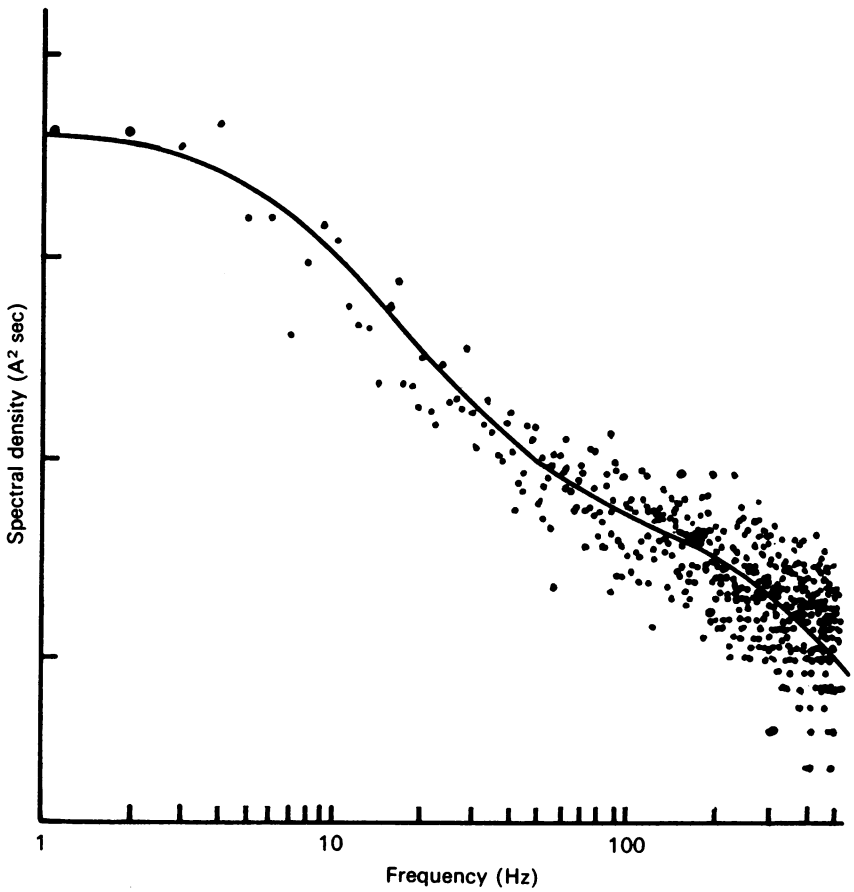

Fig. 10. Power spectrum of iontophoretically induced membrane current fluctuations, double logarithmic plot. The continuous line was calculated using mean values for G, F and α (eqns. (10), (11)and (12)) and eqn. (14). It was assumed that the conductance of the blocked state was negligible. 10 °C, −90 mV, 50 μM-QX-222. Ordinate marked in decades; lowest mark is 10^{-23} A² sec.

Macroscopic behaviour. Scheme (2) allows predictions to be made connecting the parameters of the power spectrum of microscopic fluctuations and the variables measured with single channels (cf. Ruff, 1977).

The sequential blocking scheme (2) under appropriate simplifying assumptions leads to two rates r_1 and r_2 which are given by (see Adams, 1976; Beam, 1976*b*; Ruff, 1977)

$$r_{1,2} = [\alpha + F + Gc_Q \pm \{(\alpha + F + G.c_Q)^2 - 4\alpha F\}^{\frac{1}{2}}]/2. \quad (14)$$

Under the assumption $Gc_Q + F > \alpha$ they simplify to

$$r_1 \simeq \alpha + G.c_Q + F$$

$$r_2 \simeq \alpha . \left(1 + \frac{Gc_Q}{F}\right)^{-1}.$$

Considering eqns. (7) and (8):

$$r_1 \simeq [\bar{t}_o]^{-1} + [\bar{t}_b]^{-1}$$

$$r_2 \simeq [\bar{t}_g]^{-1}.$$

In words: the fast reaction rate of noise analysis or relaxation studies is approximately equal to the sum of the reciprocals of mean block time and mean open time. The slow reaction rate is approximately equal to the reciprocal of the mean group (or burst) duration.

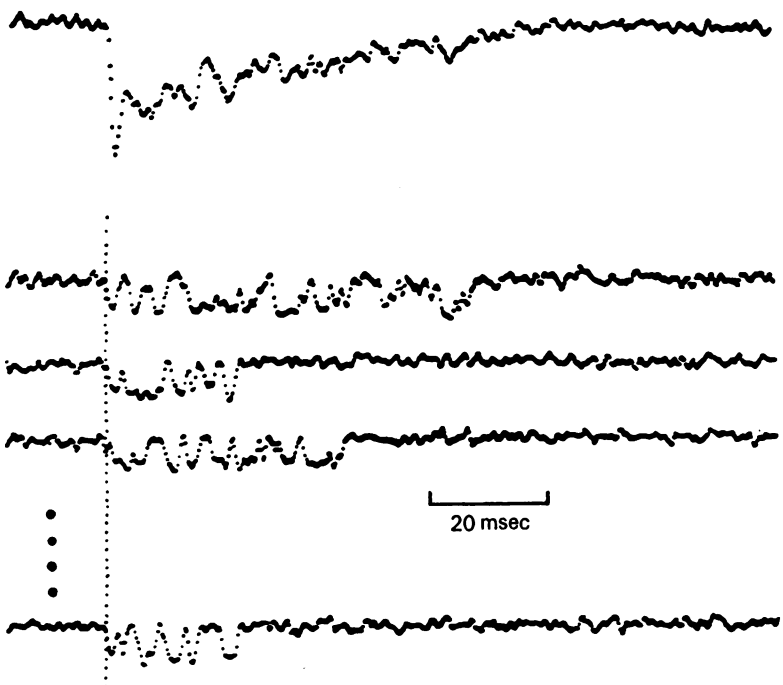

Fig. 11. Computer-generated hypothetical miniature end-plate current: 37 bursts from a fibre in 10 μM-QX-222 at -120 mV (four examples of the bursts are given in the lower section of the Figure) were superimposed on the computer after aligning their initial rising phases. The resulting wave form (uppermost trace) resembles a biphasic m.e.p.c. under the influence of QX-222 (see Ruff, 1977). In this Figure all the contributions add with their maximum value for a time period comparable to $\bar{t}_o$ (the mean open time of the short pulses). This way, a fast initial overshoot is produced. Thereafter each burst contributes only its mean value, and this second phase fades away with the mean duration of the bursts.

In order to test this relationship we performed noise analysis of iontophoretically induced membrane currents using an iontophoretic pipette which contained 1 M-sub. Standard techniques were used (cf. Anderson & Stevens, 1973; Ruff, 1977). A spectrum obtained in the presence of 50 μM-QX-222 at -90 mV is shown in Fig. 10. The

continuous line is a double Lorentzian calculated according to eqn. (14), using values for $\alpha(V)$, $G(V)$ and $F(V)$ as given by eqns. (10)–(12). The only free parameter in this fit is the zero frequency asymptote, which is proportional to the number of channels contributing to the conductance. Thus, the theory accurately predicts macroscopic membrane current fluctuations. It must be pointed out, though, that extreme caution had to be used during the iontophoresis experiments, to keep the dose of suberyldicholine and the resulting membrane currents low. At high doses, two component spectra were obtained even in the absence of local anaesthetic, which might be explained by a local anaesthetic-like effect of the agonist itself.

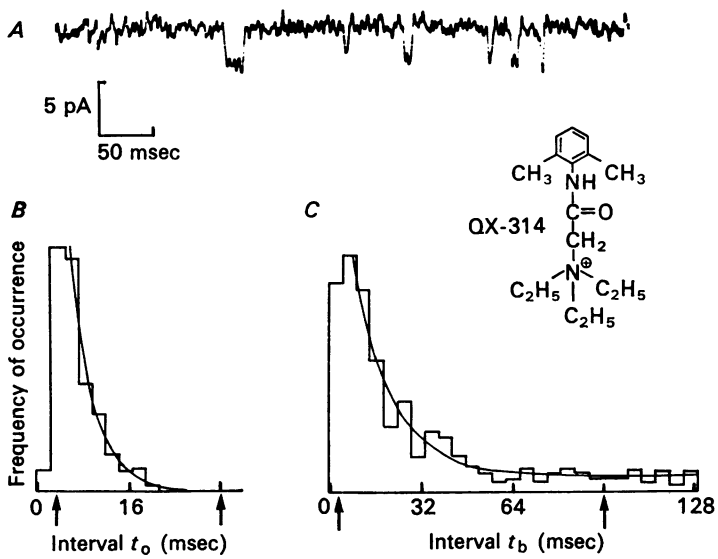

Fig. 12. The action of QX-314. *A* is a recording of membrane current comparable to Fig. 2*C*. The insert gives the structural formula of QX-314. *B*, histogram of mean open times, comparable to Fig. 5*A* (10 μM-QX-314, −120 mV, 9 °C). *C*, histogram of mean block times comparable to Fig. 5*B* (10 μM-QX-314, −100 mV, 9 °C).

A comparison of predicted and actual miniature end-plate currents (m.e.p.c.s) cannot be performed, since there are no suberyldicholine-m.e.p.c.s at the denervated preparation. However, it can be illustrated what m.e.p.c.s would look like, if they could be produced under the present conditions, assuming that a m.e.p.c. is nothing more but a superposition of currents from many synchronously opening channels. To simulate this, current contributions from thirty-seven bursts were superimposed on the computer, after aligning their initial rising phases (see Fig. 11). The resulting wave form had a double exponential decay phase very similar to m.e.p.c.s observed in the presence of QX-222.

The action of QX-314. QX-314, which is structurally very similar to QX-222 (compare inserts Fig. 1 and Fig. 12), at first sight had a completely different action on single channels. Instead of bursts of pulses only an irregular sequence of very short pulses was seen. A recording is shown in Fig. 12*A*. Such behaviour, however, would be expected if the blocked interval was long compared to the open time. This would create very long bursts of widely spaced short pulses, which could no longer be

recognized as bursts. Statistical analysis, however, should still reveal their characteristics. This was, in fact, the case. A histogram of open times, t_o, gave the blocking rate according to eqn. (7). Fig. 12*B* gives such a histogram with a mean open time of 4·2 msec (at 9 °C, −120 mV). Normalizing to 8 °C this corresponds to a forward rate constant for the blocking reaction of $1{\cdot}9 \times 10^7$ sec^{-1} M^{-1} which is almost identical to that for QX-222 at −120 mV ($2{\cdot}05 \times 10^7$ sec^{-1} M^{-1}).

The histogram of block intervals is more difficult to interpret, since it is no longer possible to distinguish the long intervals between bursts from those between pulses

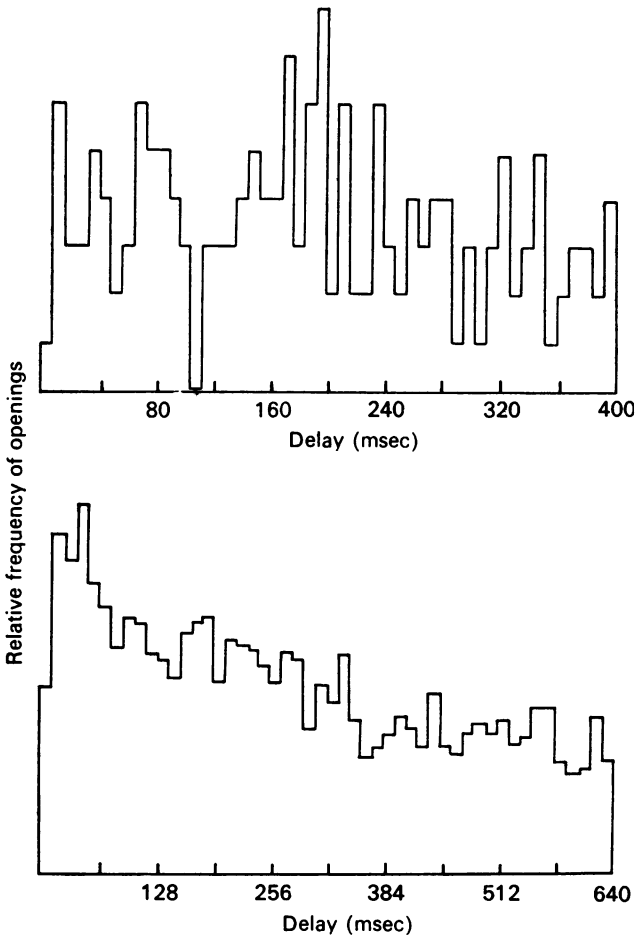

Fig. 13. Pulse correlation histograms in the absence (at −110 mV, 7 °C, part *A*) and in the presence of local anaesthetic QX-314 (at −100 mV, 9 °C, part *B*, see text).

in a burst. Also, it is no longer possible to unambiguously select data sections with non-overlapping bursts, since there is now only a small probability that multiple-conductance levels occur during overlap. Assuming, however, that the degree of overlap is small, the histogram of low conductance intervals should still approximately be a double exponential, with one time constant equal to the mean block time, and the other time constant equal to the mean interval between bursts. Fig. 12*C*

gives such a histogram, which shows a fast component with a time constant of 28 msec. The slow component is sufficiently slow to be approximated by a constant in the least square fit. This result is an indication that the mean block time is roughly 30 msec, which is an order of magnitude longer than that for QX-222 under comparable conditions. This result may be an underestimate if the condition of non-overlapping bursts was not met. In fact, the amplitude and time course (not shown in Fig. 12) of the second component indicate that there may be as much as twofold overlap, such that the true mean block time may be twice the apparent value.

Another test for consistency of the data is the analysis of pulse correlation histograms (see Methods section). These histograms give a measure for the probability that any channel openings (or unblockings) occur at time t after a given opening. For recordings in the absence of local anaesthetics, the pulse correlation histograms were flat (Fig. 13*A*), indicating that individual channels open independently from all the others. In the presence of QX-314 there should be an increased probability of channel opening (or unblocking) after a given opening (or unblocking) for a time period equal to the burst duration. This was actually found, providing an order of magnitude estimate for the burst length (230 msec $< \bar{t}_g <$ 500 msec). Within this limit of resolution eqn. (9), relating burst duration to the reaction rates of the model, was fulfilled.

In conclusion. The action of QX-314 seems to be basically very similar to that of QX-222 with almost the same blocking rate. The unblocking rate, however, is one order of magnitude smaller. Consequently the burst duration is very long. The dissociation constant, on the other hand, is therefore very low ($K_{\mathrm{Diss}} = F/G \approx 10^{-6}$ M) which makes QX-314 a much more efficient channel blocker.

DISCUSSION

Cholinergic agonists open ionic channels in the membrane of denervated frog muscle fibres, and thereby produce square pulse-like elementary current wave forms. In the presence of local anaesthetics these pulses are chopped into bursts of much shorter pulses. In the Results section the effect has been interpreted in terms of a simple sequential blocking scheme. This mechanism has an intuitive physical realization (suggested by Adams (1976, 1977) for the case of barbiturates and procaine), which postulates that the drug molecule, like any other ion, tries to pass the channel. It interacts with the channel more strongly than other ions, dwelling inside for several msecs (or tens or hundreds of msec in the case of QX-314) and thereby blocks the channel to other ions.

A number of alternative mechanisms are conceivable, however. Most of these can be eliminated from consideration. First, however, we shall simply list the experimental findings in a model-independent fashion.

Model independent statements. (I) Single channel recording allows for distinction between three channel states, which will be termed closed, open, and blocked. Although the closed and blocked states are not measurably different in their conductance they can be distinguished by their mean lifetimes. (II) After initial opening, the channel switches several times between open and blocked before finally closing.

(III) At all concentrations of QX-222 tested, only one class of channels was detected on the basis of event duration histograms. (IV) The open state in the presence of local anaesthetic has the same conductance as the open state in its absence. (V) Rates for channel closing, blocking and unblocking are voltage sensitive. Channel blocking has the reverse voltage dependence of channel closing; channel blocking and unblocking have approximately equal and opposite voltage dependences. (VI) The apparent blocking rate increases linearly with local anaesthetic concentration, while the unblocking rate is concentration independent. (VII) The total time (on the average) that a channel spends in the open state after initial opening is invariant (i.e. the sum of all the short open periods in a burst is equal to the mean open time of channels before adding local anaesthetic, which is implied in eqn. (9)).

It is apparent that several of the mechanisms suggested for the action of local anaesthetic can be ruled out by inspection of the data. Indeed, Ruff (1977) has already presented compelling evidence against these alternatives, based on properties of m.e.p.c.s and ACh-induced end-plate current noise. First, we found no evidence that the gating of separate Na^+ and K^+ selective channels changed in the presence of local anaesthetics. Second, we found no evidence that single channel currents in the presence of local anaesthetic decayed as the sum of two exponentials. We also did not see a low but appreciable blocked conductance, as suggested in the kinetic scheme proposed by Steinbach (1968*b*). At all potentials and QX-222 concentration there was only one class of channels present: we saw no evidence to support 'parallel' models in which local anaesthetic induces the appearance of several classes of receptor before receptor activation.

Instead all our data indicate that a local anaesthetic molecule transiently binds to open channels, which in the electric measurement, shows up as transient channel blockade. We arrive at this 'minimum model' (of scheme (2)) readily, if we limit our consideration to the three states that we can actually distinguish in the electrical measurement and connect them by those arrows which have an equivalent in an observable conductance-transition. Assignment of the experimentally verified properties of the transition 'open-blocked' to be bimolecular, and of the transition 'blocked-open' to be first order, completes the model of scheme (2). Furthermore, a 'hidden' transition from the blocked to the closed state is made unlikely by the observation that the length of the bursts increases with concentration of QX-222 (see Results section). As pointed out before, we cannot exclude, however, a possible transition from blocked to closed, which occurs at a rate smaller than $\alpha/10$. This qualification seems important in the light of recent findings by Adams (1977) on the action of procaine. Adams (1977) found no indications for the closing of procaine-blocked channels in his kinetic studies. However, equilibrium studies, which were done complementary to the kinetic ones, indicated that there must be such a process occurring at very slow rates. An analogous mechanism might apply here.

Even if we allow for additional states and 'hidden' transitions, there are not that many simple reaction schemes which are consistent with the data. Basically, the choice is between the simple scheme (2) and a slightly fancier version, eqn. (15), which separates the binding and blocking reactions into two steps. We shall first assume that the simple scheme holds, and see what we can make of the data.

The location of the binding site. Earlier investigations indicated that local anaes-

thetics act from the outside on acetylcholine receptors (Steinbach, 1968*a*). This conclusion, which is contrary to the effects in nerve fibres (Frazier, Narahishi & Yamada, 1970), is supported by our finding that QX effects are similar no matter whether QX is contained only in the pipette or in both the pipette and the external bath: QX concentrations at the internal surface should be widely different in the two cases, since the lidocaine derivatives are applied to quite different areas of fibre surface for different lengths of time.

The voltage dependence of the reaction rates give another clue to the location of the binding site: the equilibrium dissociation constant of QX-222 changes e-fold for a voltage change of 32 mV (eqn. (13)). Thus K_D can be written

$$K_D = K_{D,V=0} . \exp (V/32).$$

According to the simplest physical theory, the argument of the exponential should have the form (Woodhull, 1973)

$$f . V . z . e/kT.$$

In this equation f is the fraction of the membrane field sensed by the ion as it reaches its binding site, V is the membrane potential, e is the electronic charge, z is the valency of the binding ion, k is the Boltzman constant and T is the absolute temperature. With the above values an f of 0·78 is obtained, which means that the binding site is at a location where it can sense 78 % of the applied field, or that the binding site is roughly three quarters across the membrane, if a constant membrane field is assumed.

Blocking and unblocking rates have essentially equal but opposite voltage dependences, as expected if the voltage dependences result from the movement of a charged particle into and then back out of a field. These features suggest that the binding site is inside the channel, since it is hard to conceive of another binding site which has a sufficiently polar environment to interact strongly with a cation, and at the same time is in the membrane interior.

The forward rate constant for QX-binding requires some further comment. We find almost identical values (at -120 mV) of 2×10^7 M^{-1} sec^{-1} for both QX-222 and QX-314, which is within one order of magnitude of the binding rate for some barbiturates and almost identical to the binding rate of procaine (Adams, 1976, 1977). As pointed out before by Adams (1976) this value is also similar to blocking rates found for STX ($1{\cdot}01 \times 10^7$) and TTX in frog node Na^+-channels (Wagner & Ulbricht, 1975). Also, it is close to the rate of TEA blocking of squid K^+-channel (Armstrong, 1966) and to the rate of block of squid Na^+-channels by pancuronium (Yeh & Narahashi, 1977). Similar rates of block by such a large class of different substances suggests that the binding reaction is diffusion controlled.

It is hard to estimate the theoretical limit for a binding reaction of an ion inside a channel, since numerous assumptions on the surrounding medium and kinetic restrictions have to be made. However, a simple argument can be made which compares the number of blocking events per unit time with the number of ions entering the channel (Armstrong, 1966). According to Fig. 8 there are approximately 200 blocking events per second at 10 μM-QX and -120 mV, or one blocking event per 5 milliseconds. During that time there is a net inward ion flow of approximately 10^5 ions. According to the concentration ratio between QX-222 and the major current

carrier Na^+ ($c_Q/c_{Na} \approx 10^{-4}$) and the ratio of ionic mobilities (assumed to be 1/2·5), every fourth QX ion entering the channel leads to a block. Thus, the measured blocking rate is very close to the theoretical maximum.

These considerations suggest, then, that the local anaesthetic binding site is in the interior of the membrane, most likely in the lumen of the channel, and that the drug binding rate is diffusion limited.

An alternative scheme for local anaesthetic action. We cannot, however, rule out an alternative and more complex scheme for local anaesthetic action. Consider the results of separating drug binding from channel blocking:

$$\begin{array}{lll} (nT + R \rightleftharpoons T_nR) & & \text{closed} \\ \Big\uparrow\Big\downarrow & & \\ T_nR^* + QX \underset{n}{\overset{m}{\rightleftharpoons}} T_nR^*Q & & \text{open} \\ \Big\uparrow q \quad \Big\downarrow p & & \\ T_nR'Q & & \text{blocked.} \end{array} \qquad (15)$$

If we assign the observed voltage dependence to the binding/unbinding steps, then the physically most plausible location for such a binding site is in the lumen of the channel, as discussed above. However, the situation changes if the voltage dependence is assigned to the conformational changes. Indeed, our data are consistent with a version of scheme (15) in which the binding and unbinding steps are rapid and in equilibrium at all times, while the voltage sensitive conformational change steps determine the duration of blocked and unblocked periods. The best test of this particular scheme is to determine the concentration dependence of the blocking rate, since this scheme requires that the blocking rate shows a simple hyperbolic dependence on local anaesthetic concentration, Unfortunately, the available data have not been taken over a suitable concentration range so this test cannot be applied. There are two arguments, however, which make the alternative of scheme (15) an unlikely one. First, the scheme implies that the transition from the open to the blocked state has a voltage dependence opposite to that of normal channel closing. Such a molecular mechanism seems unlikely, although it is possible. Secondly, Adams (1976) has studied a class of uncharged drugs which show similar blocking behaviour without any voltage dependence. If these drugs act by the same mechanism, involving a conformational change, then the loss of the voltage dependence with uncharged drugs is unexpected, unless the conformational change carries the blocking molecules across a substantial fraction of the membrane.

There are several arguments against a mechanism according to scheme 15 with local anaesthetic binding being slow while the blocking reaction is fast. Within this scheme the blocking reaction should be shifted to the blocked side ($p > q$) for an efficient block, which we see experimentally. From that several inconsistencies follow. The first is that, if the binding is assumed to be voltage independent then the observed blocking rate should also be voltage independent. A second point is that the assumption of slow binding requires quite rapid conformational changes. The relative mean conductance of a channel with drug bound is approximately proportional to $q/(p+q)$ (the fraction of time the channel spends open while drug is bound). Thus $q \simeq 0{\cdot}05\,p$: Similarly, the observed unblocking rate will approximately equal $0{\cdot}05.n$, so $n \simeq 1{\cdot}10^4$ sec^{-1} (for meaning of p, q, n see scheme (15)). The blocking and unblocking rates must

be at least 10 times this, so $q = 10^5$ sec^{-1} and $p \simeq 2 \times 10^6$ sec^{-1} at 0 mV. These are relatively rapid rates for protein conformational changes.

We cannot rigorously exclude the possibility that local anaesthetics associate rapidly and reversibly with a binding site on the receptor and that a subsequent conformational change actually occludes the channel. The simplicity of the blocking hypothesis is, however, appealing. In addition, we have found no data that would necessitate adding the additional conformational change.

It should be pointed out that the simple scheme in which local anaesthetic molecules simply plug open channels results in a stabilization of the open state. From equilibrium thermodynamic consideration, the open state will have a higher affinity for cholinergic ligands. Thus, the simple plug theory is consistent with the data obtained by Cohen, Weber & Changeux (1974) showing that local anaesthetics increased the agonist affinity of ACh receptors on membrane fragments from *Torpedo*. It also agrees with their finding that the local anaesthetic binding site is different from the ACh-binding site, and suggests a specific location for the site – inside the channel.

Do local anaesthetics bind to closed channels? As is pointed out above, our evidence suggests a binding site in the channel, which is only accessible when the channel is open (cf. Steinbach, 1968*b*).

Beam (1976*b*) and Ruff (1977) postulated a local anaesthetic action before channel opening in addition to that described here. This additional step, in their interpretation, makes channels susceptible to the subsequent blocking action of local anaesthetics. Their data suggested the existence of normal, unmodified channels at intermediate QX-222 concentrations. We did not encounter indications of any such unmodified channels even at our low QX-222 concentrations. Consider the histogram of open times under 10 μM-QX, -120 mV (Fig. 5*A*). There are 215 open events shown in this histogram. Among them one single event is longer than half the mean open time of the unmodified channel (close to 40 msec under these conditions). Thus, the contribution of unmodified channels must be vanishingly small under the conditions of our experiments. In some of the measured histograms of mean open times there was a slight tendency for over-representation of long-lasting events. This small overabundance of long channels, if significant at all, could be a property of at most 1–5 % of all the channels in the population.

When considering these differences to earlier work, it should be emphasized that we worked with denervated, enzyme treated preparations, using sub as an agonist.

Do agonists have local anaesthetic effects? We briefly mentioned the possibility that sub might act as a local anaesthetic as well as an agonist, and thus produce noise spectra with two Lorentzian components. In addition, in a few cases pulse correlation functions for sub activated channels in the absence of local anaesthetic showed some signs of non-independence in channel openings, which were caused by groups of conspicuously closely spaced current pulses. A possible interpretation of this observation is that the groups were in fact single channels intermittently blocked by agonist molecules. The observations of Colquhoun, Dionne, Steinbach & Stevens (1975) on noise produced by some benzene-ring-containing agonists are of interest here, as well. These agonists produced spectra that usually had two Lorentzian components and also had mean single channel conductances which were significantly lower than

those for receptors activated by ACh or sub. Both of these observations could be explained if these agonists (which structurally resemble local anaesthetics) had local anaesthetic-like effects on receptors at relatively low concentrations.

These observations suggest that agonists may act as local anaesthetics and transiently block open channels with efficacies which depend on the structure of the particular agonist.

Local anaesthetic action on end-plate and nerve fibre. On first view the action of the substances used in this study on end-plate and the well known action of local anaesthetics on nerve fibre have little in common. In fact Steinbach (1968*a*, *b*) selected the lidocaine derivatives for their lack of local anaesthetic action. However, it is now clear that local anaesthetics act from the inside of nerve fibres and that the relative ineffectiveness of QX-222 and QX-314 derives from the fact that, due to their charge, these substances cannot cross the membrane (Frazier *et al.* 1970; Strichartz, 1973; Narahashi & Frazier, 1975; Hille, 1977*a*). Hille (1977*a*, *b*) has most recently treated the question of how local anaesthetics act on nerve membranes. He found that the ability of 'hydrophilic' local anaesthetics (such as QX-222 and QX-314) could most readily be explained by assuming that such drugs had great difficulty in reaching the interior of the axon from the outside. Once inside, they blocked Na currents by binding to a site reached by a hydrophilic pathway. This pathway is only available when channels are open. The simplest physical realization is to assume (cf. Fig. 8 of Hille, 1977*b*) that local anaesthetics bind to a site in the lumen of an open Na channel and occlude the pore. This picture is quite similar to the one which seems most reasonable for the action of QX-222 and QX-314 on end-plate channels.

Conclusion

All of the data we obtained by studying currents through single open channels suggest that local anaesthetics transiently occlude open channels. They appear to bind to a site about three quarters of the way through the membrane, and it is likely that a reasonably large fraction of the local anaesthetic molecules, swept into the channel, actually plug it.

This work was conducted in the laboratory of Dr C. F. Stevens at the Physiology Department, Yale University and was supported by N.I.H. grant to C. F. Stevens. We appreciate the help we received through many stimulating discussions with Drs C. F. Stevens, K. G. Beam and R. L. Ruff. J. H. S. was supported by a Muscular Dystrophy Association postdoctoral fellowship.

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