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SITES OF ACTION OF PROCAINE AT THE MOTOR END-PLATE

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SUMMARY

1. The effects of procaine at pH 7·4 and 9·9 were studied by examining the decay phase of spontaneous miniature end-plate currents (m.e.p.c.s) recorded from toad sartorius muscle fibres.

2. Following exposure to procaine (0·05–0·1 mM) at pH 7·4, the decay of m.e.p.c.s rapidly became biphasic, and could be described as the sum of two exponential components.

3. When the same concentrations of procaine were applied at pH 9·9, the development of biphasic m.e.p.c.s took much longer. Reversal of the effect upon washing out the procaine was much slower at pH 9·9 than at pH 7·4.

4. A rapid change in pH from 7·4 to 9·9 during exposure to a constant concentration of procaine quickly reduced the effect of procaine on m.e.p.c.s. The effect gradually returned after prolonged exposure to procaine at pH 9·9.

5. These results suggest that procaine applied at high pH, where it is predominantly in uncharged form, may be diffusing to a site of action which is not directly accessible from the external surface of the membrane.

6. The voltage-dependence of procaine action was similar whether it was applied at pH 7·4 or 9·9.

7. Intracellular injection of procaine rapidly produced biphasic m.e.p.c.s, whether the extracellular pH was 7·4 or 9·9. The effect of membrane potential on these m.e.p.c.s was similar to that seen for biphasic m.e.p.c.s produced by extracellular application of procaine.

8. The results indicate that procaine can affect end-plate channels when applied to either surface of the muscle membrane, and that the voltage-dependence of procaine action does not arise from the influence of membrane field on the movement of charged procaine molecules into open channels.

INTRODUCTION

At the neuromuscular junction, end-plate currents (e.p.c.s) and spontaneous miniature end-plate currents (m.e.p.c.s) normally decay with a single exponential time course. Many compounds, such as the local anaesthetic procaine, alter the decay

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phase of e.p.c.s and m.e.p.c.s, and produce double exponential (biphasic) decays. Procaine is thought to act by entering and blocking ionic channels which have previously been opened by the action of acetylcholine (Adams, 1976, 1977; Neher & Steinbach, 1978). Channel blockade by molecules of drug renders the channel non-conducting, and produces a very rapid decay phase in e.p.c.s. and m.e.p.c.s. Rapidly repeated blocking and unblocking of the channels produces a long tail in the currents until the channels finally close. This sequential model of channel blockade has been analysed in detail by Adams (1976, 1977):

$$\text{closed} \underset{\alpha}{\leftarrow} \text{open} \overset{fc}{\underset{b}{\rightleftharpoons}} \text{blocked},$$

where α, f and b are rate constants and c is the drug concentration. If b is very small, the slow component may not be visible, and currents will appear to have a single exponential time course which is much faster than control.

The actions of many charged drugs that are thought to be channel blockers are sensitive to transmembrane voltage. At the neuromuscular junction, hyperpolarization increases the effects of externally applied procaine (Kordas, 1970; Deguchi & Narahashi, 1971; Maeno, Edwards & Hashimura, 1971; Adams, 1977), QX222 and QX314 (Beam, 1976; Neher & Steinbach, 1978; Ruff, 1982), curare and gallamine (Manalis, 1977; Katz & Miledi, 1978; Colquhoun, Dreyer & Sheridan, 1979; Colquhoun & Sheridan, 1981), tetraethylammonium (Adler, Oliveira, Eldefrawi, Eldefrawi & Albuquerque, 1979), decamethonium and hexamethonium (Adams & Sakmann, 1978; Milne & Byrne, 1981), atropine and scopolamine (Adler, Albuquerque & Lebeda, 1978), amantadine (Tsai, Mansour, Eldefrawi, Eldefrawi & Albuquerque, 1978) and histrionicotoxin (Albuquerque, Kuba & Daly, 1974). Since these drugs are all positively charged at physiological pH, the movement of drug into the channel seems to be enhanced as the inside of the fibre is made more negative. The drug is able to 'sense' a fraction of the voltage drop across the membrane as it travels toward its binding site (Strichartz, 1973; Woodhull, 1973).

According to this concept, the effect of voltage should depend on whether the drug approaches its binding site from the inside or outside surface of the membrane. At the node of Ranvier and in squid axons, hyperpolarization tends to relieve block of excitable sodium channels by positively charged local anaesthetics (Narahashi, Frazier & Moore, 1972; Narahashi & Frazier, 1975; Hille, 1977*a*, *b*). In nerves, these drugs are thought to act from the axoplasmic side, rather than the external face of the membrane, and this would account for the opposite voltage-dependence to that noted at the neuromuscular junction. In fact, the voltage-dependence of block of end-plate channels by quaternary agents depends on whether they are applied on the inside or outside of the membrane (Aguayo, Pazhenchevsky, Daly & Albuquerque, 1981; but see also Horn, Brodwick & Dickey, 1980).

Uncharged local anaesthetics (Hille, 1977*a*, *b*; Ogden, Siegelbaum & Colquhoun, 1981) and barbiturates (Adams, 1976) can also interact with channels in nerve and muscle. As expected, though, their effects are relatively independent of membrane voltage. Such lipid-soluble compounds could conceivably reach their site of action, which may be inside the channel, by passing through a hydrophobic region of the membrane (Hillie, 1977*a*, *b*; Miledi & Parker, 1980).

We report here that procaine (pK_a 8·9), which exists predominantly in a charged form at normal pH, is also effective in producing biphasic m.e.p.c.s when applied in its neutral form at high pH. Uncharged procaine may be diffusing through the membrane to its binding site near the channel, or may enter the cytoplasm and act from inside the fibre. Katz & Miledi (1980) have shown that procaine produces biphasic e.p.c.s when injected intracellularly, and that the voltage-dependence of procaine action then resembles that found during extracellular application. We have confirmed and extended their results, finding a similar voltage-dependence when procaine is applied at high pH. These results show that procaine may act at a site which is not directly accessible from outside the fibre, and also suggest that the voltage-dependence of procaine action may be due to a factor other than non-specific interaction of the drug with the electric field of the membrane.

METHODS

Experiments were performed during the spring months (September–December) on isolated sartorius muscles of the cane toad *Bufo marinus*. Muscles were bathed in a Ringer solution containing 170 or 180 mM-NaCl, 2·5 mM-KCl, 1·8 mM-$CaCl_2$, and 2 mM-HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulphonic acid, pH 7·4) or CHES (cyclohexylaminoethane sulphonic acid, pH 9·9) buffer (Calbiochem). These solutions were hypertonic in order to increase spontaneous miniature end-plate potential frequency.

M.e.p.c.s were recorded focally with an extracellular electrode containing 200 mM-NaCl, 2·5 M-KCl and 1·8 mM-$CaCl_2$, or under voltage-clamp conditions. For voltage-clamp experiments, some muscles were glycerol-treated (Gage & Eisenberg, 1969) to prevent fibrillations which often occurred at high pH. Voltage electrodes were filled with 3 M-KCl, while current-passing electrodes were filled with a mixture of 0·8 M-KCl and 1·8 M-K_3 citrate. Both had resistances of about 5 MΩ. Voltage-clamp current was monitored by means of a virtual earth circuit. Bath temperature was adjusted with a Peltier cooling device.

Procaine HCl (Australian Pharmaceutical Industries) was applied by superfusion at a concentration of 0·05 or 0·1 mM, or was injected into muscle fibres by passing depolarizing current through an electrode containing 0·5 M-KCl and 1 M-procaine (pH 6). Procaine solutions were always prepared immediately before use.

Electrical signals were filtered (band-width 1 Hz to 3·5 kHz) and stored on FM tape for later analysis by computer. M.e.p.c.s which overlapped other m.e.p.c.s, or which had 10–90% rise times much greater than 200–250 μs were discarded. M.e.p.c.s (usually twenty to forty) were averaged after being synchronized in time using their 90% peak levels. The time constants (τ_D) of averaged currents obtained in the absence of procaine were determined by a least squares fit to $I(t) = I(0)\,e^{-t/\tau_D}$, where $I(t)$ is the current amplitude at time t after the peak. The decay phase of averaged m.e.p.c.s recorded in the presence of procaine was expressed as the sum of two exponential components, where the current I at time t after the peak is $I(t) = A_f e^{-t/\tau_f} + A_s e^{-t/\tau_s}$ and $\tau_f < \tau_s$. Parameters were adjusted to provide the best visual fit to the data. Rate constants derived from the blocking model (Adams, 1977) were calculated according to the equations (Gage & Sah, 1982):

$$b = \tau_D\,\tau_s^{-1}\,(\tau_f^{-1} + \tau_s^{-1}), \quad f = (\tau_s^{-1} + \tau_f^{-1} - \tau_D^{-1} - b)/c.$$

RESULTS

pH-dependence of procaine action

In the absence of procaine, a change in pH from 7·4 to 9·9 produced a slight shortening of m.e.p.c.s, seen as a reduction in τ_D (see also Takeda, Barry & Gage, 1980). M.e.p.c.s were recorded extracellularly or under voltage clamp from five cells which were exposed sequentially to solutions at the two different pHs. The average ratio

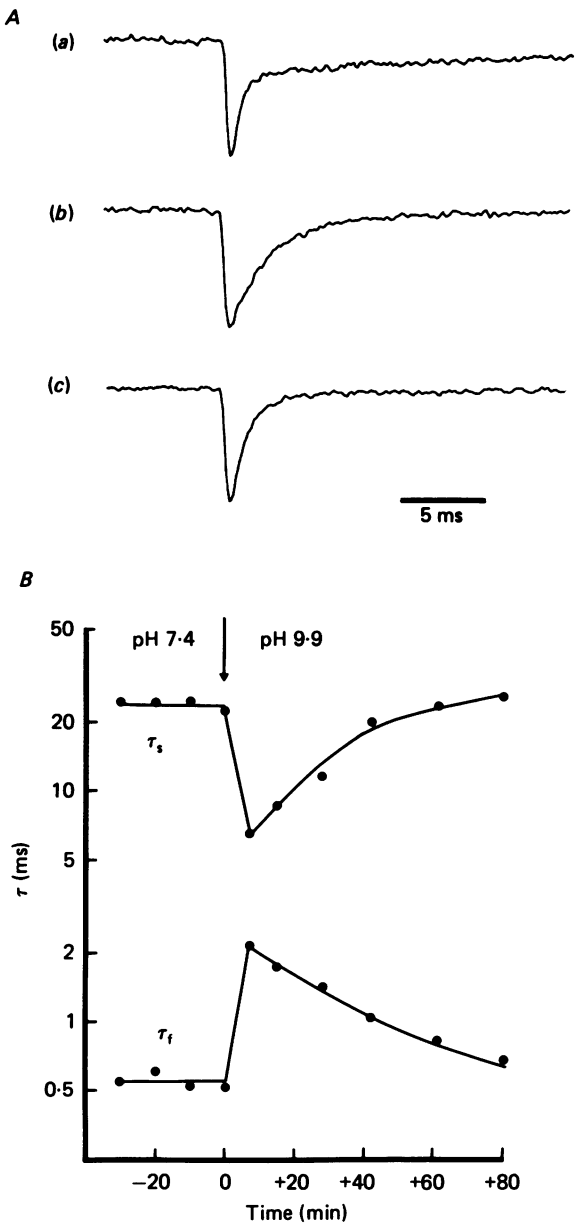

Fig. 1. Effect of a change in pH from 7·4 to 9·9 on biphasic m.e.p.c.s. The fibre was allowed to equilibrate for 50 min in 0·1 mM-procaine at pH 7·4, then the bathing solution was rapidly changed (time 0) to one containing 0·1 mM-procaine at pH 9·9. *A*, averaged m.e.p.c.s recorded extracellularly (*a*) after equilibration in procaine at pH 7·4 ($n = 37$), (*b*) immediately after the change to pH 9·9 ($n = 37$), and (*c*) 40 min later at pH 9·9 ($n = 7$). Currents have been normalized to the same peak height. *B*, m.e.p.c. decay was resolved into fast (τ_f) and slow (τ_s) time constants, which are plotted as a function of time. Temperature 17 °C.

of time constants of decay (τ_D) at pH 9·9 to pH 7·4 was 0·83 ± 0·14 (mean ± S.E. of the mean). At 10–12 °C, the average τ_D in fibres voltage-clamped at −90 mV was 6·6 ± 0·45 ms (mean of eleven ± S.E. of the mean) at pH 7·4, and 5·3 ± 0·39 ms (mean of eleven ± S.E. of the mean) at pH 9·9 ($0·05 < p < 1$ by Student's t test). An e-fold change in τ_D was produced by a change in voltage of 81 ± 4·5 mV at pH 7·4 and 76 ± 2·1 mV at pH 9·9, indicating that the voltage sensitivity of τ_D is unaffected by the pH change ($p > 0·3$). A slight reduction in τ_D was also observed at pH 5·4 compared with τ_D at pH 7·4. Although others have reported that frog m.e.p.c.s or e.p.c.s are greatly affected by pH (Scuka, 1975; Mallart & Molgo, 1978), the effects seem to be species-dependent (Landau, Gavish, Nachshen & Lotan, 1981). The effects of smaller changes in pH within the range 7·4 to 9·9 were not investigated.

During exposure to procaine at pH 7·4 at the resting membrane potential, m.e.p.c. decay was obviously biphasic, with two very distinct components (Maeno, 1966; Gage & Armstrong, 1968; Kordas, 1970; Deguchi & Narahashi, 1971; Maeno *et al.* 1971; Katz & Miledi, 1975). Fig. 1 illustrates the effect of changing the pH from 7·4 to 9·9 while maintaining a constant procaine concentration. As soon as the bathing solution was changed, the two components became less obvious, and the time constants tended to converge. However, the procaine effect gradually returned, and eventually the time constants were similar to those observed before the pH change. The relative amplitude of the fast component also became larger at high pH, suggesting an increase in the blocking effect of procaine. After about 60 min, the slow component was barely visible, and m.e.p.c. decay was adequately described by one very fast time constant.

The initial decrease in apparent potency suggests that an active form of procaine is being removed, or washed out, as the pH is changed from 7·4 to 9·9. Since the charged form predominates at pH 7·4, but is present only at a very low concentration at pH 9·9, the decrease in local anaesthetic effect is probably due to a reduction in the concentration of the charged species in the extracellular solution.

At pH 9·9, 91 % of the procaine molecules are in an uncharged (neutral) form. The neutral form of the drug may diffuse through a hydrophobic region of the membrane to an area where procaine can again have an effect on m.e.p.c. decay. Procaine may be reaching the inside of the muscle fibre (Ohki, Gravis & Pant, 1981), where it could act in either its charged or uncharged form, or may be approaching a site of action from within the membrane.

Fig. 2 also demonstrates the presence of a 'barrier' to procaine action when it is applied at high pH. At pH 7·4, procaine acted within a few minutes, and its effect remained fairly constant for as long as the procaine concentration was maintained. Changes in m.e.p.c. decay were readily reversible when procaine was removed, although the recovery rate was slightly slower than the time course of the initial effect (Adams, 1977). At pH 9·9 (Fig. 2*B*) the separation of the two phases took much longer to develop, and the difference between τ_f and τ_s, the time constants of the fast and slow phases respectively, continued to increase as long as the procaine application was maintained. On washing out the procaine, recovery was extremely slow and incomplete, even after 2 h of perfusion with drug-free solution (not shown).

The actions of procaine at high pH, which occur only after a considerable time delay, could conceivably be due to a slow conversion of neutral procaine to the

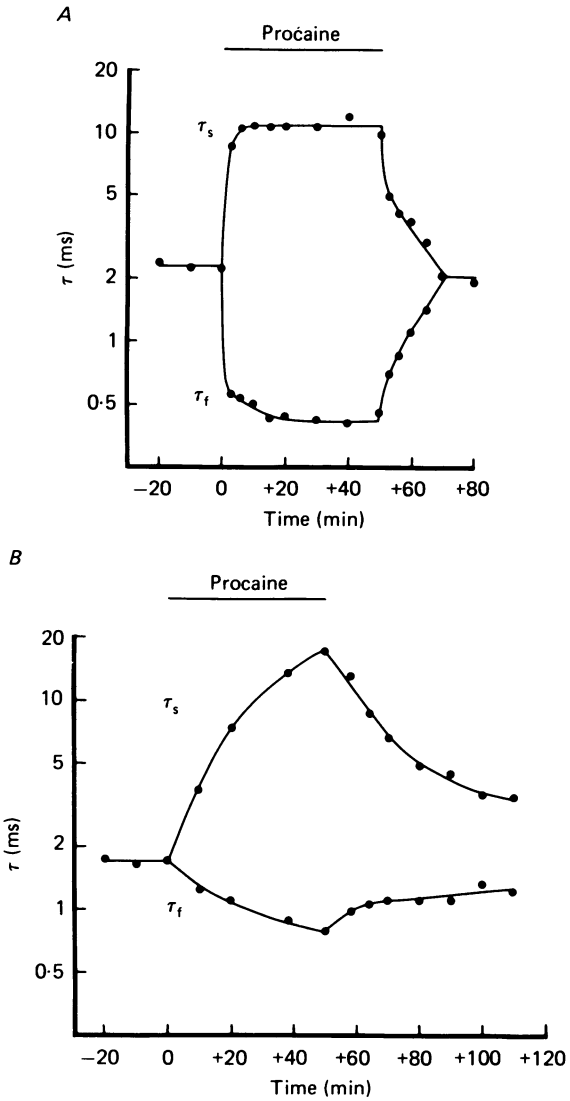

Fig. 2. Time course of procaine action. Procaine (0·1 mM) was rapidly applied at time 0, and was removed from the solution 50 min later. Fast and slow time contants (τ_f and τ_s) of m.e.p.c. decay are plotted as a function of time. *A*, pH 7·4; *B*, pH 9·9. The muscle was equilibrated to the appropriate pH for 50 min before procaine application. The perfusion rate was the same for both pHs. *A* and *B* represent different end-plates. Each point is derived from the average of twenty to eighty extracellularly recorded m.e.p.c.s. Temperature 20 °C.

charged species. If the external Ringer solution were not well buffered, then the pH in the immediate vicinity of the membrane could possibly be decreasing with time. If the pH were to approach 8·9, the pK_a of procaine, an appreciable fraction of procaine near the membrane would become charged. Such charged molecules which formed in the region of end-plate channels could then act to block channels from the outside, as normally occurs at lower pH.

This explanation is unlikely, however, for several reasons. Time constants and the relative amplitudes of the two phases measured at pH 9·9 continued to change with prolonged exposure to procaine, and did not level off to correspond to values measured at pH 7·4. One would expect no further change in τ_f and τ_s to occur once the majority of procaine molecules had acquired a positive charge. Procaine actually became more effective at pH 9·9 than at 7·4 if the exposure period were sufficiently long. Furthermore, if the slow effect at pH 9·9 were due to a gradual decrease in pH, then the effects of procaine should wash off rapidly. The speed at which m.e.p.c.s return to normal after washing should be the same as occurs at pH 7·4 when procaine is removed from the solution. The finding that the procaine effect was fairly slow to wash out at high pH again argues against the possibility that the time course of procaine action reflects local changes in pH outside the membrane. Finally, the perfusion system was able to change the solution near the membrane surface at a high rate, as indicated by the rapid onset of procaine action at pH 7·4 (Fig. 2*A*). It seems unlikely that a sufficiently high concentration of protons would be able to accumulate in order to alter the pH by almost a full unit.

The slow time course of procaine action at high pH must therefore reflect the presence of some kind of barrier between the external solution and a procaine binding site. This barrier may be the muscle membrane, through which uncharged molecules of procaine must penetrate to reach their site of action.

Voltage-dependence of procaine action

It has been suggested (Adams, 1977) that the voltage-dependence of the action of procaine is related to the influence of the membrane field on the movement of procaine from the extracellular solution into an end-plate channel. In order to explore further the similarities and differences between the effects of charged and uncharged procaine, m.e.p.c.s were recorded from muscle fibres voltage-clamped at different membrane potentials to study the effects of pH on the voltage sensitivity of the procaine-blocking reaction.

Fig. 3*A* illustrates m.e.p.c.s recorded at three different membrane potentials in the presence of procaine at pH 7·4. As the membrane was hyperpolarized from −50 to −110 mV, the fast component became slightly faster and larger, while the slow component became much slower and smaller. At +50 mV, only a single time constant was required to describe m.e.p.c. decay, and this was similar to that measured from m.e.p.c.s at the same potential in the absence of procaine (Gage & Armstrong, 1968; Kordas, 1970; Deguchi & Narahashi, 1971; Maeno *et al.* 1971).

The voltage-dependence of procaine action is shown in more detail in Fig. 3*B*, where fast and slow time constants are plotted as a function of voltage. Using the sequential model, measured values of τ_f and τ_s were used to calculate rate constants f and b for the blocking and unblocking reaction at membrane potentials in the range −50 to −130 mV (Fig. 4). For simplicity, rate constants were assumed to vary exponentially with voltage, although their observed voltage-dependence was more complex than this. At −90 mV, f was $1{\cdot}2 \times 10^7$ s^{-1} mol^{-1} and increased e-fold for a change in membrane potential of 197 mV, while b was 380 s^{-1} and decreased e-fold for a change of 152 mV (from fits to averaged data from ten to twelve fibres). The voltage-dependences of f and b were approximately equal in magnitude but opposite in sign,

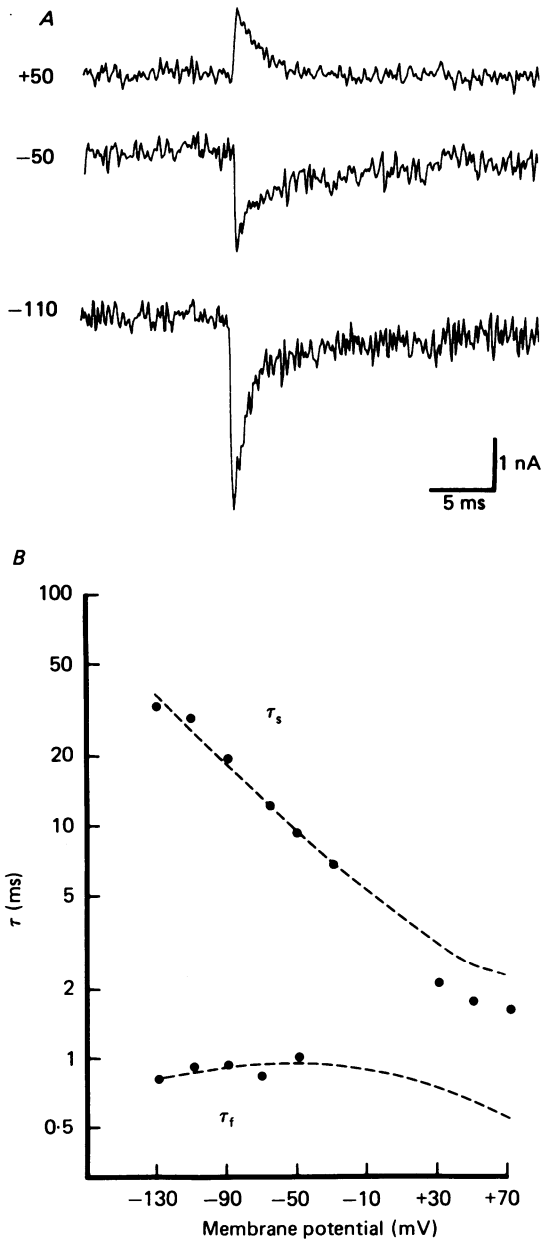

Fig. 3. Effect of membrane potential on biphasic m.e.p.c.s at pH 7·4. *A*, individual m.e.p.c.s recorded in a fibre voltage-clamped at +50, −50 and −110 mV in the presence of 0·05 mM-procaine. *B*, τ_f and τ_s plotted as a function of membrane potential. Each point is the time constant of the average of eleven to sixty-four m.e.p.c.s. The dashed lines show the predicted behaviour of τ_f and τ_s based on the voltage-dependences of f and b measured between −50 and −130 mV, and assuming an exponential dependence of f and b on membrane potential. Temperature 12 °C.

and the equilibrium dissociation constant for the blocking reaction, $K = b/f$, thus had a potential dependence of 86 mV per e-fold change. Using this value, a binding site for procaine can be estimated to be about 30 % of the way across the membrane field (Woodhull, 1973). These values are in rough agreement with those reported by Adams (1977) using voltage-jump experiments and bath-applied suberyldicholine, in which K decreased e-fold for 50 mV hyperpolarization, and a procaine binding site was estimated to be 50 % through the membrane field.

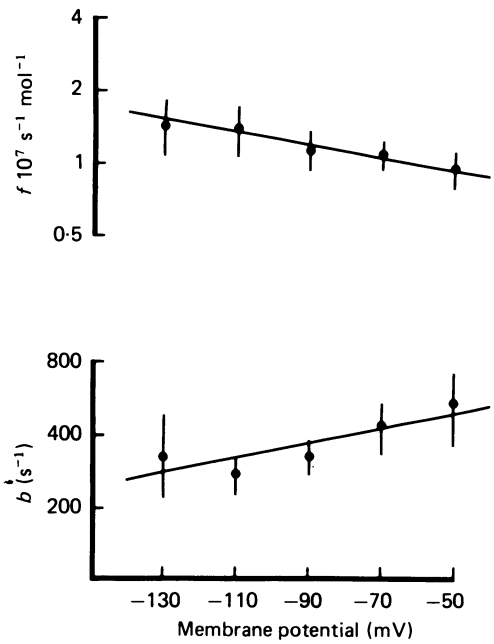

Fig. 4. Effect of voltage on the rate constants f and b for the blocking and unblocking reactions in the sequential model (see text). The lines are least squares fits, and the slopes correspond to 197 mV per e-fold change in f and 152 mV per e-fold change in b. Each point is the mean from ten to twelve fibres. Errors bars show ±S.D.. Temperature 10–12 °C; pH 7·4.

However, these values for the voltage-dependences of f and b, which were calculated between −50 and −130 mV assuming an exponential dependence on voltage, do not accurately predict the decay of m.e.p.c.s at positive potentials. In Fig. 3*B*, calculated curves based on measured values of f and b are superimposed on the data points. At +30 to +70 mV, m.e.p.c. decay should still be biphasic, with two unequal time constants. Currents at positive potentials appear to be single exponentials, with a time constant intermediate between the predicted τ_f and τ_s. Possible explanations for this discrepancy include the possibilities that the outward movement of ions through the channel is dislodging procaine from its binding site, similar to the effects of K^+ current on tetraethylammonium-blocked channels in axons (Armstrong, 1966), or that f and b are not simple exponential functions of voltage.

This reasoning assumes that procaine is acting according to the blocking model to enter channels from the outside of the membrane. According to this model, procaine acting from inside the fibre or from within the membrane, as may occur at high pH,

should have a voltage-dependence which is quite different to that observed at pH 7·4.

At pH 9·9 (Fig. 5), the voltage-dependence of procaine action was not different from that at pH 7·4. Procaine became much more effective with hyperpolarization, as seen previously. Inward currents were biphasic, while outward currents occurring at depolarized potentials remained single exponentials. Specific values for f and b could not be determined because c, the drug concentration near procaine's site of action, could not be measured. The voltage-dependences of f and b were also difficult to determine, because of the large variability between experiments. Complete current–

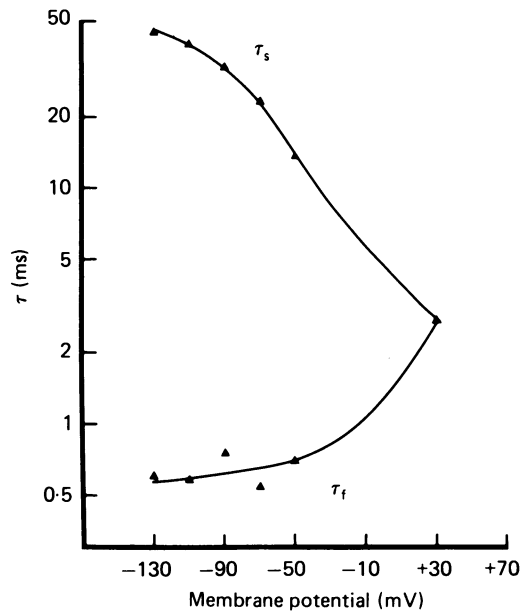

Fig. 5. Voltage-dependence of the decay of biphasic m.e.p.c.s at pH 9·9. M.e.p.c.s were recorded in a voltage-clamped fibre 30 min after application of 0·1 mM-procaine at pH 9·9. Fast and slow time constants (τ_f and τ_s) are plotted as a function of membrane voltage. Each point represents the time constant of the average of twelve to sixty-five m.e.p.c.s. Temperature 12 °C. Lines have been drawn by eye.

voltage relationships were obtained in only three out of six fibres, since all data had to be obtained very quickly. The procaine effect continued to increase with time, and the slow component eventually became so small, especially at hyperpolarized potentials, that τ_s could not be estimated. In addition, some measured values of τ_f and τ_s were not consistent with the predictions of the blocking model, and calculated values of f and b thus had no real significance. Nevertheless, it is obvious that these data are qualitatively similar to those obtained at pH 7·4, and that the effects of procaine at high pH are enhanced by hyperpolarization.

Unfortunately, these results do not help to clarify the active form or site of action of procaine applied at high pH. If uncharged procaine were diffusing into the muscle fibre and blocking channels from the inside in its charged form, then one would expect the voltage sensitivity to be opposite to that seen at pH 7·4. Assuming that charged

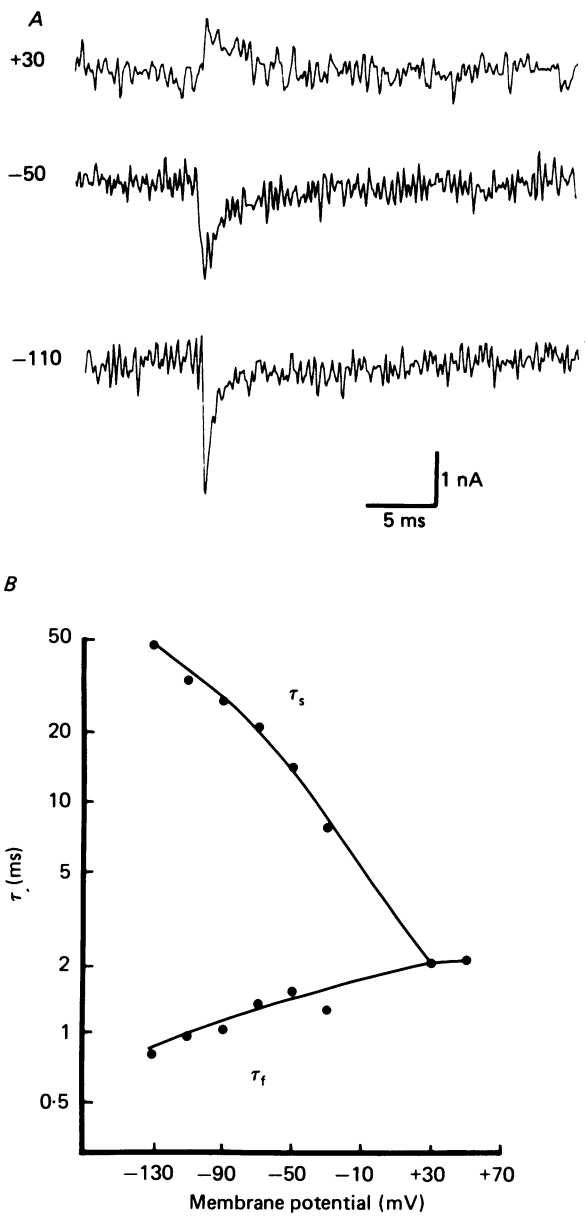

Fig. 6. Effect of membrane potential on biphasic m.e.p.c.s produced by intracellular injection of procaine. Procaine was injected into individual fibres by passing depolarizing current through an intracellular electrode containing procaine. *A*, individual m.e.p.c.s recorded at +30, −50 and −110 mV. *B*, fast and slow time constants (τ_f and τ_s) plotted as a function of membrane potential. Each point is the time constant of the average of two to thirteen m.e.p.c.s. Lines have been drawn by eye. Temperature 12 °C, external pH 9·9.

molecules of procaine are affected by the voltage drop across the membrane, depolarization should help drive positive charges into the channel and increase the blocking effect. If procaine were acting in a neutral form, either directly from the membrane lipid environment or from the muscle interior, then its action should be relatively insensitive to the membrane field.

Intracellular injection of procaine

As a further confirmation of the findings at high pH, procaine was injected intracellularly into single voltage-clamped muscle fibres. M.e.p.c.s rapidly became biphasic following the passage of depolarizing current through a procaine-containing electrode (see Fig. 6). Hyperpolarization increased the blocking effect of procaine, while outward currents observed at depolarized potentials were virtually indistinguishable from control. The voltage-dependence observed with intracellular injection appeared to be identical to that obtained during external application at both pH 7·4 and 9·9, though specific values for f and b could not be determined.

Procaine injected inside muscle fibres was most probably acting from the inside, and not from the outside of the membrane. Assuming an internal pH of 7 inside the fibre (Roos & Boron, 1981), only about 1 % of the injected procaine would be uncharged. If this 1 % were to diffuse to the outside of the fibre and ionize, it could act to block channels from the external surface. In order to exclude this possibility. the external pH was maintained at 9·9 to prevent accumulation of charged procaine outside the fibre. Since procaine applied externally at pH 9·9 took some time to become effective (Fig. 2*B*), and the effects of intracellular injection were immediately apparent, it is doubtful whether intracellularly injected procaine was acting from outside the fibre.

DISCUSSION

These results raise some rather interesting questions regarding the actions of procaine at the neuromuscular junction. It has generally been believed that charged molecules of procaine act from the external side of the membrane to enter and block channels which have previously been activated by acetylcholine (but see Katz & Miledi, 1980). We have found that procaine can also interact with channels via a route which does not involve direct access from the external surface of the membrane. In addition, the voltage-dependence of this interaction appears to be independent of the route by which procaine reaches this site of action.

At high pH, externally applied procaine must cross some type of diffusion barrier to reach its binding site, and may be acting from the inside of the fibre. Uncharged procaine may diffuse across the muscle membrane, which should be extremely permeable to the drug (Ohki *et al.* 1981), and reach the fibre interior, where the pH is about 7 (Roos & Boron, 1981). Procaine inside the fibre would be converted to its charged form, and the concentration gradient for entry of neutral procaine into the fibre would be maintained. The amount of charged procaine inside the fibre may therefore build up to substantial levels, and procaine applied at high pH may thus be working from inside the fibre. Since a binding site for procaine has been estimated to be 30–50 % through the channel (Adams, 1977), procaine may be able to reach this

same site from either end of the channel. The effectiveness of procaine injected inside muscle fibres supports the idea that the drug may be working from the inside.

Two binding sites

Aguayo *et al.* (1981) have found that quaternary derivatives of phencyclidine and piperocaine are effective in blocking channels when applied from either the inside or outside of the muscle membrane. They suggest that separate binding sites for these agents may be present on both the inner and outer aspects of the ionic channel. Procaine may similarly bind to two sites within the channel, each accessible from a different side of the membrane.

However, the possibility of two binding sites is complicated by the finding that other charged compounds which block acetylcholine channels from the outside are ineffective when applied intracellularly. Tetraethylammonium, atropine methyl bromide, curare, gallamine, QX222 and QX314 (Horn *et al.* 1980; Katz & Miledi, 1980; Aguayo *et al.* 1981) do not alter e.p.c. decay when injected inside muscle fibres, indicating that they do not react with binding sites that are readily accessible from the inside of the membrane. If a channel did indeed contain two binding sites, one on each side of the membrane, then the pharmacology of the two sites must be different. This suggests that there may be quaternary compounds which block channels from the inside but which are ineffective when applied extracellularly. It also implies that drugs which are effective when applied intracellularly and those which are ineffective must recognize different functional groups on their binding sites. This means that their binding sites on the external side of the membrane would not be identical.

Access via a lipid environment

An alternative explanation for the effectiveness of procaine at high pH is that procaine may act via the lipid region of the membrane. Miledi & Parker (1980) have suggested that D600, which is also effective in blocking acetylcholine-activated channels when applied from either side, may penetrate the lipid phase of the membrane and bind to a site associated with the acetylcholine receptor–channel complex. Similarly, uncharged molecules of procaine could approach their site of action through the membrane lipids, and react with a binding site near the channel–membrane interface. Charged procaine injected into the fibre could also be acting in this manner. Although the amount of uncharged procaine inside the fibre would be only about 1 % of the total procaine present, there may be enough of the uncharged form to diffuse into the membrane from the inside. This idea is supported by the finding that the quaternary agents QX222 and QX314 are ineffective when injected intracellularly (Horn *et al.* 1980).

Voltage-dependence

It was thought that knowledge of the voltage-dependence of procaine action at high pH would help determine whether procaine acts from inside the fibre in charged form, or in the lipid environment in neutral form. In the first case, procaine should have the opposite voltage-dependence to that seen at pH 7·4, where hyperpolarization is thought to aid the movement of positively charged procaine into the channel. In the

second case, the effect of procaine should be essentially independent of membrane voltage.

However, the voltage-dependence of procaine action is similar no matter how the drug is reaching its site of action. This finding is incompatible with the theory that procaine moves into the membrane channel under the influence of the membrane field. One possible explanation for this finding is that uncharged molecules of procaine which pass through the lipid matrix can react with protons donated by membrane proteins, thus becoming ionized near the channel. The charge form of procaine would still be the active species, but the observed voltage-dependence at high pH would reflect the movement of protons within the membrane down the imposed electrical gradient.

Alternatively, the voltage-dependence of procaine action may arise from changes in membrane structure which occur during hyperpolarization. If the conformation of the binding site were to change with membrane voltage, thus altering the affinity of the site for procaine, then the voltage-dependence of procaine action might not be related to its method of application. It is clear that modifications in the sequential model of channel blockade will be needed to explain all of these effects of procaine.

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