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COMPARISON OF DESENSITIZATION AND TIME-DEPENDENT BLOCK OF THE ACETYLCHOLINE RECEPTOR RESPONSES BY CHLORPROMAZINE, CYTOCHALASIN B, TRITON X-100 AND OTHER AGENTS

ROGER ANWYL[1] & TOSHIO NARAHASHI

Department of Pharmacology, Northwestern University Medical School, 303 E. Chicago Avenue, Chicago, IL 60611, U.S.A.

1 Chlorpromazine, cytocholasin B, Triton X-100, lidocaine, QX-314, pentobarbitone, heptanol and ethanol, but not benzocaine or methylguanidine, caused a time-dependent inhibition of acetylcholine (ACh) potentials. Increasing the concentration of the agents increased the rate of inhibition.

2 The recovery rate from the time-dependent inhibition was the same as from desensitization except in Triton X-100 which slowed the recovery.

3 Hyperpolarizing the membrane potential caused an increase in the rate of the time-dependent inhibition.

4 It is suggested that the time-dependent inhibition and desensitization are very similar phenomena, with either ACh (in desensitization) or an agent (in time-dependent inhibition) causing a block of the activated ACh receptor, and with dissociation from the binding site being very slow.

5 Many of the agents also cause a steady state inhibition of the ACh receptor which appears to be caused by a separate blocking action of the agents.

Introduction

It has been postulated that desensitization is increased by agents such as chlorpromazine, SKF 525A and mesphenal (Magazanik & Vyskocil, 1973; 1977). As little is known about the mechanism of desensitization or how these agents increase desensitization, it was decided to investigate the action of a number of agents in relationship to desensitization.

The results of the study show many agents cause a time-dependent inhibition of acetylcholine (ACh) receptor responses which is very similar in several respects to the desensitization caused by ACh. It is suggested that these agents and ACh cause a similar block of the activated ACh receptor, with dissociation of ACh or the agent from the receptor or channel being very slow.

Methods

These were similar to those described in the previous paper.

Some experiments were also carried out on voltage clamped end plates of frog sartorious muscle. The voltage clamp method was a standard two-microelectrode system (Takeuchi & Takeuchi, 1959).

Cytochalasin B, Triton X-100, acetylthiocholine, acetylcholine and concanavalin A were obtained from Sigma, tetramethylammonium and phenyltrimethylammonium from Pfaltz and Bauer, and lidocaine and QX-314 from Astra Pharmaceutical Products. Chlorpromazine and sodium pentobarbitone were gifts from Smith, Kline and French, and Abbott Laboratories, respectively.

In the results, the average concentration of agents decreasing the half-time of the time-dependent inhibition ($T_{\frac{1}{2}}$) by 50% are given ± 1 s.e.

Results

Many of the agents investigated inhibited the iontophoretic ACh potentials evoked from denervated rat muscle in two ways. Firstly, certain agents caused an increasing inhibition of successive ACh potentials evoked at frequencies above 0.1 to 0.5 Hz. This effect

[1] Present address: Department of Physiology, Trinity College, Dublin 2, Ireland.

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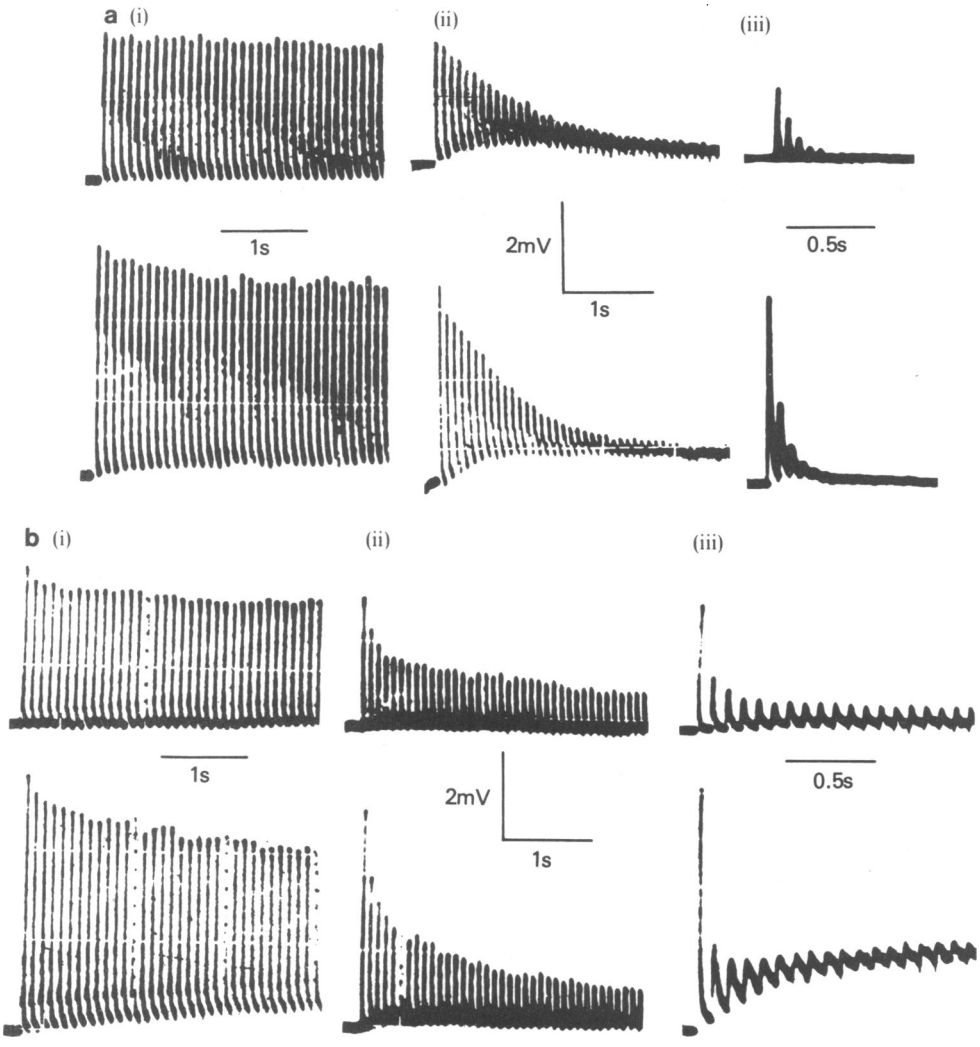

Figure 1 (a) (i) Desensitization of approximately 4 mV (top trace) and 8 mV (bottom trace) acetylcholine (ACh) potentials evoked at a frequency of 10 Hz. The potentials declined eventually to zero amplitude. (ii) and (iii) Time-dependent inhibition of the 10 Hz evoked ACh potentials superimposed on the desensitization caused by Triton X-100 at concentrations of (ii) 0.001% and (iii) 0.005%. (b) (i) Desensitization of 4 mV (top trace) and 8 mV (bottom trace) ACh potentials evoked at 10 Hz. (ii) and (iii) Time-dependent inhibition of the ACh potentials caused by chlorpromazine at concentrations of (ii) 1.5 μM and (iii) 3 μM.

will be termed time-dependent inhibition. Secondly, certain agents caused a constant reduction of ACh potentials evoked at all frequencies. This effect will be termed steady-state inhibition.

As described in the previous paper, successive ACh potentials increasingly decline in amplitude when evoked at 10 Hz, due to desensitization of the ACh receptors. Perfusion of chlorpromazine, cytochalasin B, Triton X-100, lidocaine, QX-314, sodium pentobarbitone, ethanol and heptanol were found to increase the rate at which successive ACh potentials declined in amplitude when evoked at 10 Hz (Figure 1a, b), i.e. the agent inhibition was superimposed on desensitization.

Chlorpromazine, cytochalasin B and Triton X-100 were the most potent agents at increasing the rate of

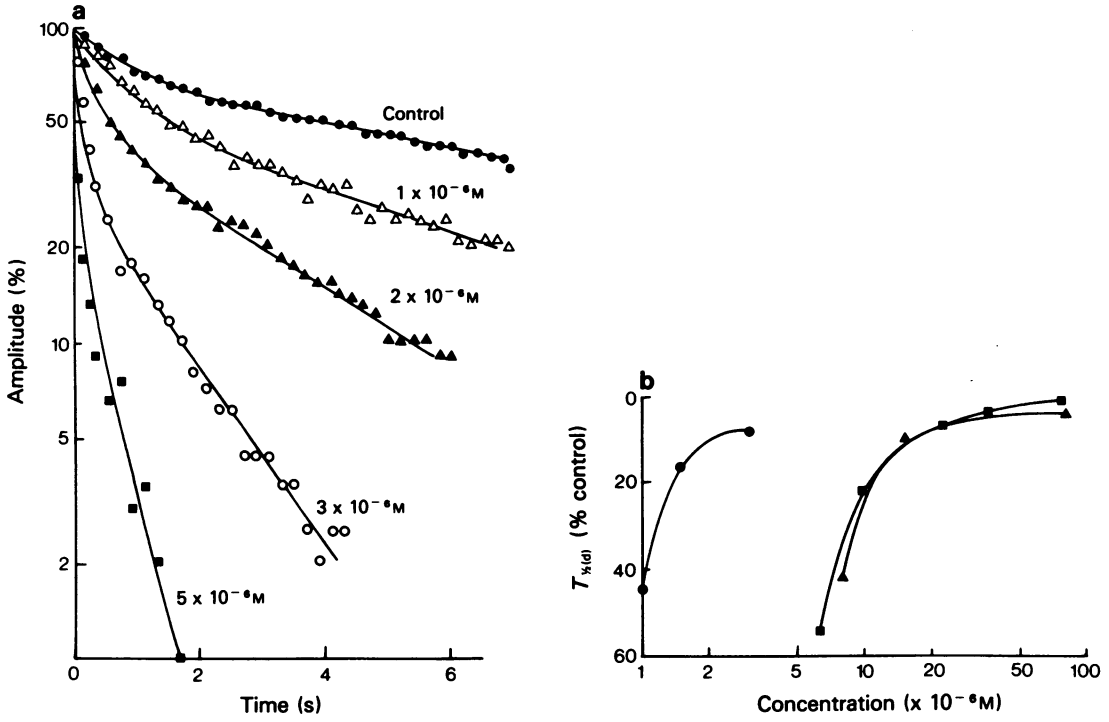

Figure 2 (a) Desensitization of acetylcholine (ACh) potentials evoked from extrajunctional ACh receptors of rat soleus muscle and the time-dependent inhibition produced by the following concentrations of chlorpromazine: 1×10^{-6} M (△), 2×10^{-6} M(▲), 3×10^{-6} M(○) and 5×10^{-6} M (■); Control (●). (b) Dose-response relationship for the time dependent inhibition produced by chlorpromazine (●), cytochalasin B (■) and Triton X-100 (▲). The time-dependent inhibition is expressed as the half-time of the inhibition as a percentage of the desensitization occurring in normal saline.

decline of the amplitude of the ACh potentials and were therefore studied in most detail. At low concentrations, these three agents increased the rate of decline of the ACh potentials up to nine fold without altering the steady-state amplitude of the ACh potentials evoked at low frequencies (up to about 0.5 Hz). At 5 to 10 times higher concentrations, these three agents also decreased the steady-state amplitude of the ACh potentials.

Figure 2a shows an example of the reduction of the amplitude of successive ACh potentials in different concentrations of chlorpromazine, each control ACh potential having a steady-state amplitude of 8 mV. The half-time of reduction of amplitude, $T_{\frac{1}{2}}$, was decreased from 3.0 s in normal saline to 1.6, 0.6, 0.15 and 0.03 s in 1, 2, 3 and 5×10^{-6} M chlorpromazine respectively. Similar reductions in amplitude of successive ACh potentials were caused by cytochalasin B and Triton X-100. Figure 2b shows a dose-response relation for the decrease in $T_{\frac{1}{2}}$ of 8 mV amplitude control ACh potentials in Triton X-100, cytochalasin B and chlorpromazine. The concentration of these agents which decreased $T_{\frac{1}{2}}$ by 50% was 1 ± 1 μM ($n = 10$) for chlorpromazine, 5 ± 1 μM ($n = 11$) for cytochalasin B (Figure 3) and 6 ± 1.5 μM ($n = 9$) for Triton X-100.

The steady-state amplitude of the ACh potential was reduced 50% by 6 ± 2 μM ($n = 6$) chlorpromazine, 60 ± 8 μM ($n = 5$) cytochalasin B, and 50 ± 1.5 μM ($n = 9$) Triton X-100.

Perfusion of chlorpromazine, cytochalasin B (Figure 3) or Triton X-100 caused a rapid reduction in the steady-state amplitude of the ACh potentials, with a maximum effect being reached after less than 5 min. However, the effect of the agents on the time-dependent inhibition was much slower, usually starting 5 min after the start of perfusion, and reaching a maximum only after 20 to 30 min perfusion. Upon washing with normal saline, the steady-state inhibition recovered to its original amplitude within 5 to 10 min, whereas the time-dependent inhibition returned to its original rate only after 15 to 30 min.

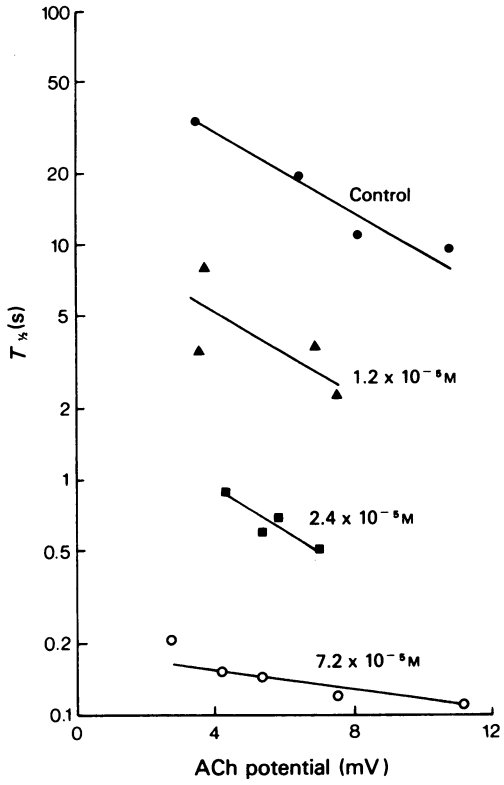

Figure 3 Half times of desensitization (●) and the half-times of time-dependent inhibition in the following concentrations of cytochalasin B: (▲) 1.2×10^{-5} M, (■) 2.4×10^{-5} M, (○) 7.2×10^{-5} M.

Lidocaine, QX-314, heptanol, ethanol and pentobarbitone were also found to increase the rate at which successive ACh potentials declined in amplitude when evoked at 10 Hz although they were much less potent than chlorpromazine, cytochalasin B and Triton X-100. The half-time of reduction of amplitude of ACh potentials was decreased 50% by 50 ± 6 μM ($n = 4$) lidocaine, 50 ± 5 μM QX-314, 150 ± 20 μM ($n = 4$) pentobarbitone, 500 ± 20 μM ($n = 4$) heptanol, and about 100 mM ethanol.

Lidocaine (Figure 4), QX-314, heptanol and pentobarbitone caused a steady state reduction of the amplitude of the ACh potentials at the same concentrations as that causing the time-dependent inhibition. However, ethanol was found to increase the steady state amplitude of the ACh potentials. Thus 100 mM ethanol increased the amplitude of the ACh potential by about 50%.

Methylguanidine and benzocaine did not cause any time-dependent inhibition but did reduce the steady state amplitude of the ACh potential. Thus the steady state amplitude of the ACh potential was decreased 50% by 500 ± 200 μM ($n = 4$) methylguanidine, and 450 ± 300 μM ($n = 4$) benzocaine.

Recovery from time-dependent inhibition

Recovery from time-dependent inhibition was studied by evoking ACh potentials at 1.5 to 4.0 s intervals during the recovery period (Figure 5a). The recovery half-time was the same as from desensitization except in Triton X-100.

The recovery half-time measured 4.2 ± 0.6 s. in 1.2 to 7.2×10^{-5} M cytocholasin B (8 experiments) (Fig. 5a), 4.8 ± 1.1 s in 1 to 5×10^{-6} M chlorpromazine (8 experiments), 4.5 ± 0.8 s in 3 to 8×10^{-5} M lidocaine (6 experiments), 3.9 ± 0.4 s in $0.5 \times 2 \times 10^{-5}$ M pentobarbitone (5 experiments), 4.1 ± 0.9 s in 6 to 8×10^{-4} M heptanol (3 experiments) and 4.0 ± 1.3 s in 10^{-1} M ethanol (3 experiments). Altering the concentration of these agents did not alter the recovery half-time.

In Triton, X-100, the rate of recovery was much slower (Figure 5a, b). The recovery half-time averaged 9.0 ± 1.8 s (6 experiments) in 0.0005% Triton X-100, 11.8 ± 1.8 s (6 experiments) in 0.001% Triton X-100 and 12.6 ± 1.9 (6 experiments) in 0.005% Triton X-100. These represent increases in the recovery half-time of 2.1, 2.7 and 3.0 fold in 0.0005, 0.001 and 0.005% Triton X-100, respectively, compared with the control.

Time-dependent inhibition in the absence of desensitization

When ACh potentials are evoked at low frequencies of up to about 1 Hz, they remain a constant amplitude in normal saline. However, successive ACh potentials were found to be increasingly inhibited when evoked at frequencies of 0.5 to 1 Hz in the presence of chlorpromazine, cytochalasin B, lidocaine, QX-314, sodium pentobarbitone, ethanol and heptanol. In the presence of these agents, ACh potentials of constant amplitude could only be evoked at frequencies of less than about 0.1 to 0.5 Hz.

Recovery half-time from the time-dependent inhibition varied from 4 to 5 s except in Triton X-100 when the recovery rate was slowed 2 to 3 times.

Kinetics of time-dependent inhibition

The kinetics of the reduction in amplitude of successive ACh potentials caused by the agents, whether in the presence or absence of desensitization, was very similar to that of desensitization. Thus there was a

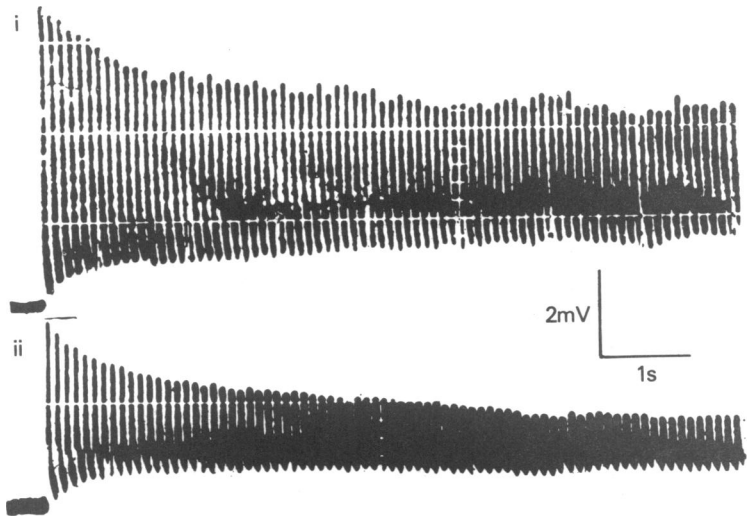

Figure 4 Desensitization in normal saline (i) and the time-dependent inhibition in 50 μM lidocaine (ii).

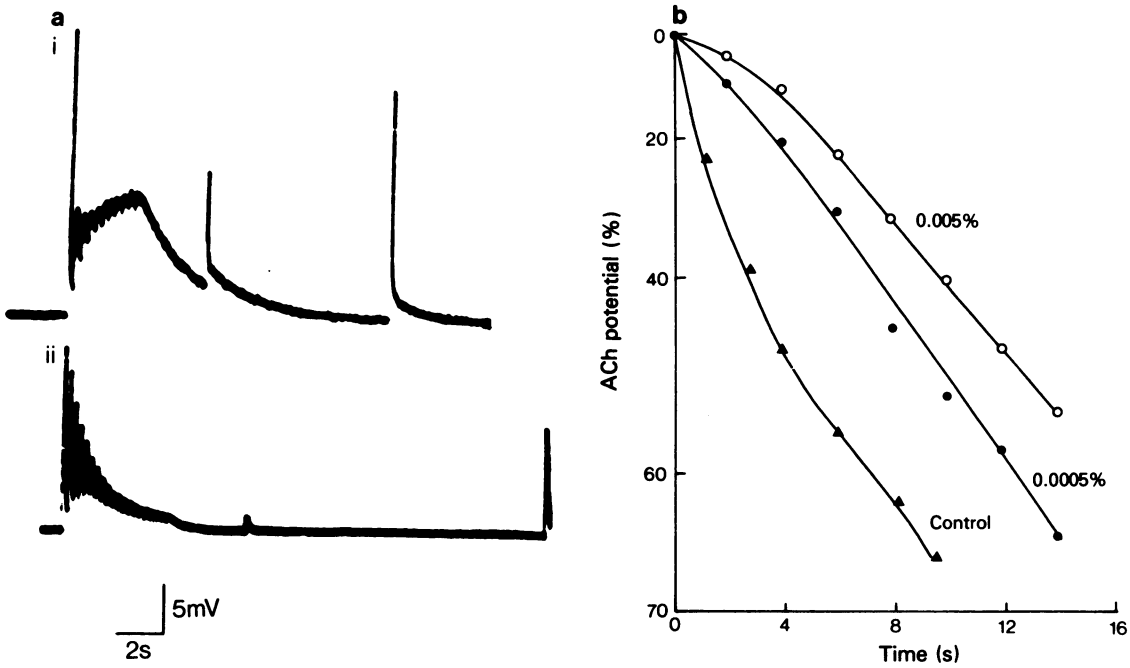

Figure 5 (a) Onset and recovery of time-dependent inhibition in (i) 2×10^{-6} M chlorpromazine, (ii) 0.001% Triton X-100. Note the slow rate of recovery in Triton X-100. (b) Recovery from desensitization in normal saline (▲) and from time-dependent inhibition in 0.0005% (●) and 0.005% (○) Triton X-100. Each point is the average taken from the experiments in which 2 to 3 acetylcholine potentials were evoked during each recovery period.

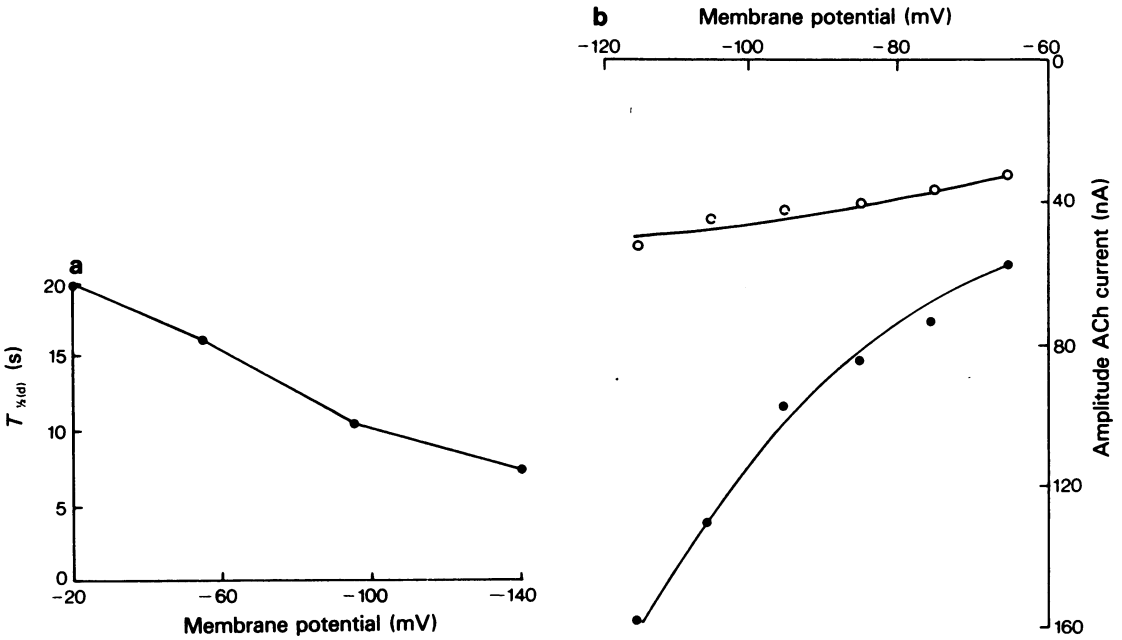

Figure 6 (a) The values of the half-time of the time-dependent inhibition produced by 5×10^{-7} M chlorpromazine at the frog neuromuscular junction at different clamped membrane potentials. (b) Current–voltage relationship for the normal acetylcholine (ACh) current (●) and for the ACh current after time-dependent inhibition by 5×10^{-7} M chlorpromazine (○). The current–voltage plot for the normal ACh current shows a large increase in conductance upon hyperpolarization, whereas the plot for the inhibited ACh current shows a small decrease in conductance upon hyperpolarization.

very rapid initial decline of the potentials, followed by a slower decline. Empirically, the slower decline could be fitted by one exponential function, and the initial rapid decline by two more exponential functions.

Effects of membrane-potential on time dependent inhibition

These experiments were carried out on voltage-clamped frog endplates. In normal saline, iontophoretic ACh currents remained at constant amplitude when evoked at a frequency of 1 Hz. However, in the presence of 5×10^{-7} M chlorpromazine, successive ACh currents declined in amplitude to reach a steady state inhibited amplitude. The value of the half-time of the time-dependent inhibition, $T_{\frac{1}{2}}$ averaged 25 ± 6 s ($n = 7$) at a holding potential of -70 mV. Hyperpolarizing the membrane potential from -10 to -150 mV caused an increase in the rate of the time-dependent inhibition. The value of $T_{\frac{1}{2}}$ decreased by $5\% \pm 3\%$ ($n = 6$) per 10 mV hyperpolarization (Figure 6a).

The current–voltage relationship for the ACh currents was measured both for the control and during a train of ACh currents in the presence of chlorpromazine at a time when the ACh currents have reached a steady state inhibited value. The potential was held at different values for several seconds during the train of ACh currents. The current–voltage relationship for the normal ACh current showed an increase in slope conductance when the membrane was hyperpolarized from -10 mV to -150 mV, whereas the current–voltage relationship for the inhibited ACh current was linear or even showed a small decrease in conductance upon hyperpolarization (Figure 6b).

The mean reversal potential obtained by extrapolation (2 experiment) or direct reversal (1 experiment) of the inhibited ACh current was -12 ± 2 mV, similar to that of the normal ACh current, -14 ± 3 (3 experiments).

Discussion

Chlorpromazine, cytochalasin B, Triton X-100, lidocaine, QX-312, pentobarbitone, ethanol and heptanol have all been found to cause a time-dependent inhibition of the ACh receptor responses. This was

observed both as decline in amplitude of successive ACh potentials evoked at sufficiently low frequency so that in normal saline they would remain at constant amplitude, and also as an increase in the rate of desensitization-induced reduction of ACh potentials evoked at a high frequency. This time-dependent inhibition is a very similar phenomenon to desensitization. Thus increasing the concentration of the agent or of ACh increases respectively the rate of time-dependent inhibition and desensitization. Moreover, the recovery rate from desensitization is the same as from the time-dependent inhibition caused by agents (except Triton X-100), and altering the dose of ACh or the agent (except Triton X-100) does not alter the recovery rate. In addition, the voltage dependence of the time-dependent inhibition is identical to that of desensitization. Thus the half-time of the time-dependent inhibition caused by chlorpromazine increased by 5% per 10 mV hyperpolarization, while Lambert, Spannbauer & Parsons (1977) found that the time constant of desensitization increased by 6% per 10 mV hyperpolarization.

One possible theory to reconcile the great similarity between desensitization and the time-dependent inhibitory action of the agents is that both events involve a similar block of the activated ACh-receptor complex. Thus after ACh (A) has bound to the ACh receptor (R) and caused opening of the ionic channel, it is proposed that the activated receptor (R*) is converted to an inactive non-conducting state (AR'X) by either binding of the agent (in time-dependent inhibition) or by binding of further ACh (in desensitization). The inactivation may be caused by the agent and/or ACh actually blocking the ionic channel.

The inhibition can be represented by the model (Adams, 1976; Neher & Steinbach, 1978):

$$A + R \overset{\text{fast}}{\rightleftharpoons} AR^* + X \overset{\text{slow}}{\rightleftharpoons} ARX'$$

X is a molecule of the agent or ACh. The association and dissociation of X would be slow to account for the slow onset and recovery rates of both desensitization and time-dependent inhibition.

Biochemical studies have shown that the desensitized receptor has a higher affinity than the normal receptor for ACh i.e. ACh becomes tightly bound to the receptor (Weber, David-Pfeuty & Changeux, 1975; Sugiyama, Popot & Changeux, 1976 see also Katz & Thesleff, 1957). Moreover, local anaesthetics and detergents increase this biochemical desensitization (Sugiyama *et al.*, 1976). In the above sequential scheme, ARX' would be the high affinity 'desensitized' receptor. However, it is also possible that X dissociates very quickly from ARX', leaving ACh bound to a high affinity form of the receptor, AR. The slow and constant time course of recovery would then be given by the dissociation of ACh from this high affinity receptor, the latter then returning rapidly to its normal state.

$$ARX' \overset{\text{fast}}{\rightleftharpoons} X + AR \overset{\text{slow}}{\rightleftharpoons} A + R \overset{\text{fast}}{\rightleftharpoons} R.$$

It is suggested that many agents have both a steady-state and a time-dependent inhibitory action, with the two types of inhibition being associated with different binding sites on the ACh receptor/channel. This proposal is supported by the findings that, firstly after perfusion of agents, the steady state inhibition reaches a maximum in 5 min, but the time-dependent inhibition only in 15 to 30 min. Secondly, Triton X-100, chlorpromazine and cytochalasin B produce a time-dependent inhibition at 5 to 10 times lower concentration than steady state inhibition. Thirdly, certain agents such as methylguanidine and benzocaine produce a steady-state but not a time-dependent inhibition, while ethanol causes a time-dependent inhibition although increasing the amplitude of the ACh potentials. Fourthly, the recovery rate from time-dependent inhibition is orders of magnitude slower that from the steady state inhibition.

The inhibition referred to as the steady state inhibition in this paper has been investigated in detail for several agents similar to those used in the present study. Thus octanol and atropine shortened the life time of endplate channels (Gage, McBurney & Van Helden, 1978) while local anaesthetics chopped the pulse-like elementary currents into bursts of shorter pulses. However, noise studies have shown that both the desensitization caused by ACh (Anderson & Stevens, 1973), and the inhibition of ACh responses by low concentrations of Triton X-100 (Fischbach & Laas, 1978) do not result in a change of either the single channel conductance or of the noise power spectrum. However, it is postulated this blocking action is so slow that the cut off frequency would be below the range of most noise analysis. A very similar blocking action has been found for curare on the molluscan ACh receptor (Ascher, Marty & Nield, 1978). Although the agents which cause a time-dependent inhibition have a great variety of structure, they are characterized by large hydrophobic radicals such as aromatic rings or long aliphatic chains, and also usually by a charged nitrogen atom. This suggests that both hydrophobic and hydrophilic bonding is important in the attachment of the agents to the binding site. Thus neither benzocaine, a neutral anaesthetic lacking a charged nitrogen atom, or methylguanidine, which has a charged nitrogen atom, but lacks large hydrophobic groups, causes time-dependent inhibition. Very hydrophobic agents lacking a charged nitrogen atom, such as heptanol and pentobarbitone, do cause a time-dependent inhibition, but only at very high concentrations. Triton X-100 was the only agent lacking a charged nitrogen atom found

to be very potent at causing a time-dependent inhibition, and this was the only agent to cause a different recovery rate from that of desensitization.

QX-314, which is the permanently charged form of lidocaine and therefore cannot cross cell membranes, was found to cause time-dependent inhibition in the present study. This demonstrates that the time-dependent inhibitory site is accessible from the external medium. The delay of 15 to 30 min before the time-dependent inhibition reaches a maximum suggests that the agents may bind hydrophobically to membrane lipids before they bind to the receptor or channel.

The current–voltage relationship for the normal ACh current was found to show an increase in conductance when the membrane was hyperpolarized. This increase in ACh effectiveness upon hyperpolarization has also been observed by Dionne & Stevens (1975). However, during the time-dependent inhibition, the increase in conductance upon hyperpolarization is reduced or abolished. Such a change in the current–voltage relationship is consistent with hyperpolarization causing an increase in the time-dependent inhibition, providing no change occurs in the reversal potential of the inhibited current.

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