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The mechanism of steroid anaesthetic (alphaxalone) block of acetylcholine-induced ionic currents

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1 The effects of the steroid anaesthetic alphaxalone on acetylcholine (ACh)-induced ionic channels were studied in voltage clamped 'myoballs' in culture.

2 Alphaxalone produced a reversible blockade of the ACh-evoked inward current, $ED_{50} = 6.0\ \mu M$.

3 The ACh reversal potential (−5.0 mV), the single channel conductance (13.5 pS) and mean open time (3.6 ms) were unchanged by the drug. Thus, alphaxalone produced an 'all or none' block of the ionic channel.

4 In double pulse conditioning experiments, alphaxalone produced an additional inhibition with a time constant of recovery (550 ms) much longer than the time constant of recovery of the normal desensitization (250 ms).

5 It was concluded that alphaxalone blocks active (open) ionic channels.

Introduction

Since the first observations by Cashin & Moravek (1927) that a colloidal suspension of cholesterol injected intravenously can produce anaesthesia sufficiently deep to permit major surgery, the concept of steroid anaesthesia has become well established (for review see Atkinson *et al.*, 1965; Phillipps, 1974; Gyermek & Soyka, 1975; Holzbauer, 1976). A hypnotic ability is common to most steroid hormones and synthetic steroids (Selye, 1941) and the development of steroid anaesthetics was fostered by their favourable therapeutic index. Alphaxalone (3α-hydroxy, 5α-pregnane-11, 20-dione) is a short acting synthetic steroid anaesthetic. Its neuropharmacological mechanisms have been studied in central synapses (Richards & Hesketh, 1975; Richards & Smaje, 1976; Smaje, 1976; Archer *et al.*, 1977; Morris, 1978; Scholfield, 1978; Richards & White, 1981), at the neuromuscular junction (Torda & Gage, 1977; Torda & Murphy, 1979; Pennefather *et al.*, 1980; Pennefather & Quastel, 1980) and in artificial membranes (Connor *et al.*, 1974; Lawrence & Gill, 1975). Two types of molecular action of alphaxalone have been suggested – an effect on membrane fluidity (Torda & Gage, 1977) or a more specific block of membrane ionic channels (Pennefather & Quatell, 1980).

We have described, in this paper, the effects of alphaxalone on the acetylcholine (ACh)-induced response in voltage clamped 'myoballs' (Fischbach & Lass, 1978) in culture. The molecular action of alphaxalone was studied by acetylcholine 'noise' analysis and double pulse conditioning experiments. It is suggested that alphaxalone produces an 'all or none' block of open ionic channels.

Methods

Cell culture and electrophysiological techniques and the analysis of ACh-induced current fluctuations have been described in preceding papers (Fischbach & Lass, 1978). Briefly, spherical multinucleated myoballs were prepared by allowing mononucleated precursor cells to fuse in suspension culture. The cells were mechanically dissociated from 11 day-old chick embryo pectoral muscles and were maintained in Earl's Minimum Essential Medium (EMEM) supplemented with horse serum (10% v/v) and chick embryo extract (2% v/v) in culture dishes coated with Sylgard (Dow Corning). Collagen-coated cover slips (18 mm) with attached myoballs were placed in a double-bottomed recording chamber (vol. = 0.7 ml) and continuously perfused at a rate of $2\ ml\ min^{-1}$. The medium bathing the cells was kept at room temperature (22–24°C). The cells were viewed from below with a long working distance, phase contrast objective.

Solutions

Normal EBSS (Earle's Balanced Salt Solution) contained (mM): NaCl 116, KCl 5.3, NaH_2PO_4 1.0, $MgSO_4$ 0.8, glucose 5.5, HEPES 20. The pH was 7.3.

Alphaxalone was kindly given to us by Glaxo. A stock solution of alphaxalone in ethanol was added to the EBSS. The final concentration of ethanol was 0.2% v/v and had no effect on the ACh receptor. In dose-response experiments, alphaxalone was added to the growth medium (see above) to increase the drug concentration to 300 μM.

Electrophysiology

ACh-induced transmembrane currents were recorded in voltage clamped myoballs. Control of membrane potential was achieved with two relatively blunt intracellular electrodes, one for measuring the membrane potential and the other for supplying the feedback current required to hold the membrane potential constant. Each electrode measured 3–10 MΩ when filled with 3 M KCl. The voltage clamp amplifier was built in our laboratory. The gain of the feedback amplifier was usually kept at 5,000 and the cell was clamped to −50 mV unless otherwise stated. The level of medium in the recording chamber was lowered to about 2 mm to minimize capacitive coupling between the electrodes and the bath.

For ACh noise measurements, a third electrode, filled with 2 M ACh was positioned 50–100 μm away from the cell and ACh was ejected by prolonged positive pulses. Ionophoretic electrodes had resistance in the range of 10–20 MΩ and required backing currents of 5–15 nA. The ACh electrode was located far away from the myoball to minimize variation in ACh concentration at the cell surface and to avoid the possibility of local saturation of the receptors. Slow, diffusion limited ACh responses allowed computation of the relationship between mean ACh current and the variance in current fluctuations during a single application. The relationship was always linear. No change in the membrane potential of clamped myoballs was observed during pulses of ACh large enough to generate mean inward currents of 100 nA.

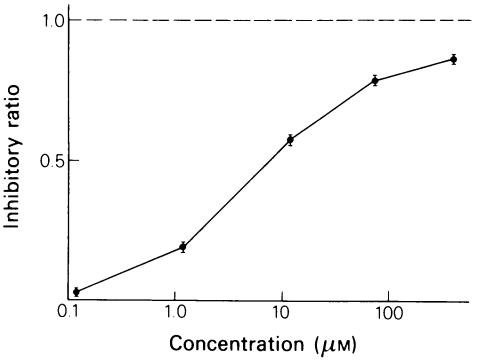

Figure 2 Dose-response curve of alphaxalone-induced reduction in ACh sensitivity in cultured chick myoballs. Cells were clamped to −50 mV and the temperature was 25°C. The inhibition ratio was defined as (Sc − Sal)/Sc where Sc is the control sensitivity and Sal is the sensitivity in the presence of alphaxalone in the same cell. Each value is the mean of at least 5 determinations and the vertical lines indicate s.e.mean.

Feedback currents were stored on magnetic tape (HP 3964, frequency-response 0–1,250 Hz). Two recording channels were used: a low gain, d.c. channel for recording the mean membrane current and a high gain a.c. channel for recording the membrane current fluctuations. An active filter (Krohn Hite 3321, band-pass usually set at 2–450 Hz) was used to prevent a changing error in the spectral density estimates. The single channel conductance (γ) and open time (τ) were calculated from the spectral density of the ACh-induced membrane current fluctuations (Katz & Miledi, 1972; Anderson & Stevens, 1973).

For ACh sensitivity measurements, a high resistance (50–70 MΩ) ACh electrode was used. These electrodes were selected for a linear voltage-current relationship up to 200 nA, and required only 2–5 nA backing current. The tip of the ACh electrode was located 10–20 μM from the exposed membrane of the myoball. (Dreyer, *et al.*, 1978). The ionophoretic

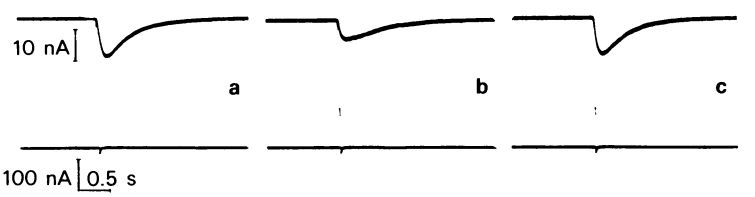

Figure 1 Reduction of acetylcholine (ACh) sensitivity by 24 μM alphaxalone in cultured chick myoball. The cell was clamped to −50 mV and the temperature was 25°C. The upper trace is the ACh-induced membrane current and the lower trace is the ionophoretic current. The control (a) sensitivity was 10.6 nA nC^{-1}. Alphaxalone (b) reduced the ACh sensitivity to 5.2 nA nC^{-1} and the effect was fully reversible; sensitivity returned to 10.0 nA nC^{-1} (c).

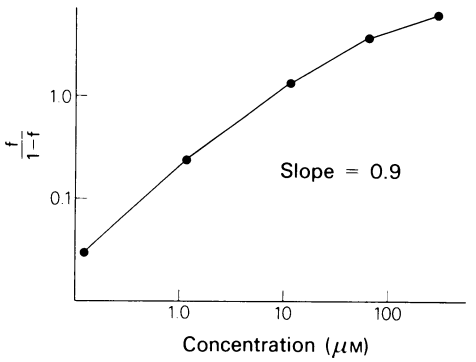

Figure 3 Hill plot of the data shown in Figure 2. At ED_{50}, the slope is nearly 1.

current was measured across a 100 kΩ resistor connected between the stimulator and ground. The electrode stray capacitance was compensated for by a variable capacitor connected across the 100 kΩ resistor. Numerical results were expressed as mean ± standard error (s.e.) of the mean.

Results

Figure 1 shows the reversible blockade of the ACh-induced response by 24 μM alphaxalone. The ACh sensitivity was defined as the peak ACh-induced inward current (nA) divided by the charge ejected from the ionophoretic electrode (nC). For the same ionophoretic current, alphaxalone reduced the ACh-induced inward current by 55%.

A dose-response curve of the alphaxalone blocking action is shown in Figure 2, and the Hill plot of the same data is shown in Figure 3. The ED_{50} of the drug was 6.0 μM and from the slope of the Hill plot ($\simeq 1$) it was assumed that one alphaxalone molecule per channel is required to block the ACh-induced current.

A typical voltage-current relationship of the ACh-induced response is shown in Figure 4. Although alphaxalone (24 μM) reduced the ACh-induced response by 60%, the ACh reversal potential (-5.0 ± 2.1 mV, $n = 6$) was unchanged. Thus, the blocking action of alphaxalone could not be explained by a change in the elctrical driving force or a change in the ionic selectivity of the channel.

Spectral analysis of the ACh-induced noise (Katz & Miledi, 1972; Anderson & Stevens, 1973) is shown in Figure 5. The control single channel conductance (γ) and mean open time (τ) were: $\gamma = 13.5 \pm 1.5$ pS ($n = 6$) and $\tau = 3.6 \pm 0.5$ ms ($n = 6$) at holding potential of −50 mV and at 24°C. At alphaxalone concentrations of 6–24 μM, which reduced the ACh sensitivity by 50–70%, the single channel parameters were: $\gamma = 13.0 \pm 1.3$ pS ($n = 6$) and $\tau = 3.7 \pm 0.4$ ms ($n = 6$).

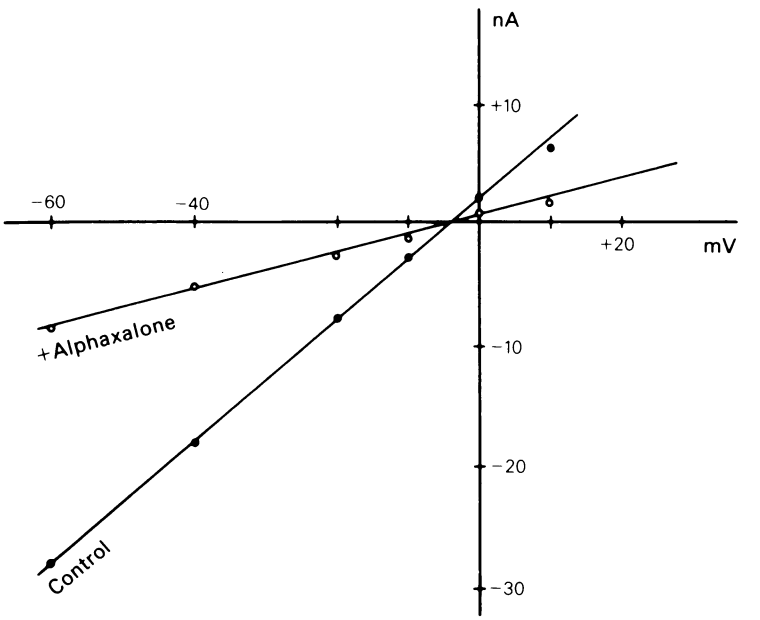

Figure 4 Voltage-current relationship of the acetylcholine (ACh)-induced current. At membrane potentials between −60 mV and + 10 mV, the ACh-induced response was reduced by 24 μM alphaxalone. However, the ACh reversal potential (−4 mV) was unchanged.

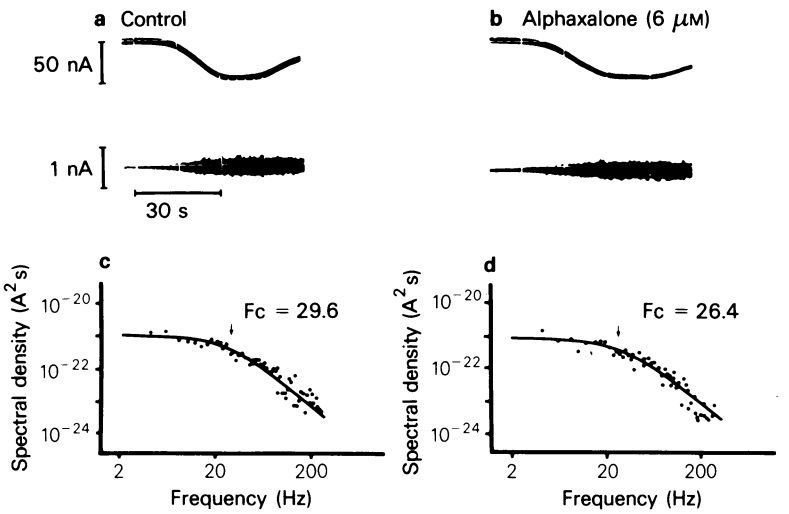

Figure 5 Acetylcholine (ACh) 'noise' analysis in cultured chick myoballs. The upper traces are the ACh-induced inward currents. In the presence of alphaxalone (b), a much larger ionophoretic current was required to produce a response comparable to the control response. The middle traces show the filtered (2–450 Hz) ACh-induced current displayed at relatively high gain. The lower traces (c and d) show the spectral density of the current fluctuations. The single channel conductance (13.5 pS) and open time (3.6 ms) were unchanged before and after alphaxalone in the same cell.

The same results were obtained at membrane potentials between −25 to −80 mV and when ACh was applied either by ionophoresis or by addition to the bathing solution. Since the channel parameters were unchanged, it was concluded that alphaxalone reduced the number of open channels and the blocking action is 'all or none' at the molecular level.

Figure 6 shows the results of a double pulse conditioning experiment (Adams, 1976; Katz & Miledi, 1978; Adams & Feltz, 1980). In (a) (control), 4 pairs of responses to ionophoresis were superimposed. The time interval between the conditioning response (cond.) and the test responses (t_1–t_4) varied between 200 to 800 ms. In the control, a slight desensitization was observed when the pulse separation was 200 ms. No desensitatization was observed when the pulse separation was increased to 400 ms or more. When alphaxalone (60 μM) was added to the same cell, all responses were depressed, and an additional inhibition became apparent even 800 ms after the conditioning pulse. The results of a similar experiment in another cell ar plotted in Figure 7. The control graph represents the time constant of recovery from the receptor desensitization (175 ms), whereas in the presence of alphaxalone the time constant increased to 540 ms. In 4 cells, the time constant increased from 250 ± 45 ms to 550 ± 30 ms. In 2 additional cells, no desensitization was observed in control, but in the

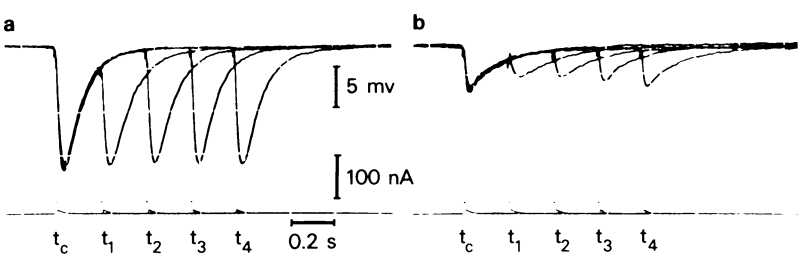

Figure 6 Double pulse conditioning experiment. The upper trace shows a superposition of 4 pairs of acetylcholine (ACh)-induced responses, control (a) and in the presence of 60 μM alphaxalone (b). The lower trace shows the ionophoretic current. In the presence of alphaxalone, the ACh-induced response is depressed, and an additional ('extra') inhibition is observed even 800 ms after the conditioning response.

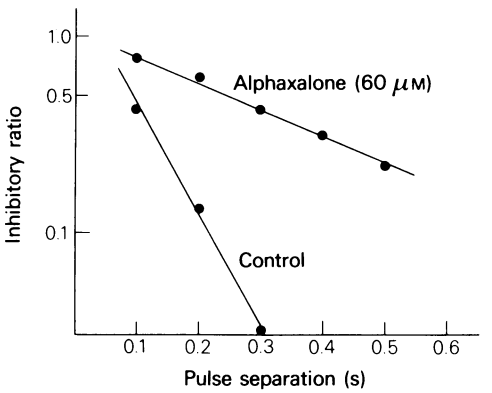

Figure 7 'Extra' inhibition produced by alphaxalone in a double pulse conditioning experiment similar to that illustrated in Figure 6. Here, the time interval increment was 100 ms rather than the 200 ms in Figure 6. In control, the time constant of decay of the normal desensitization was 275 ms. Alphaxalone increased the time constant to 540 ms.

presence of alphaxalone, an additional inhibition with the same time constant became apparent.

The additional ('extra') inhibition produced by alphaxalone was also demonstrated in an experiment in which the ACh dose was gradually increased (Figure 8). In the presence of alphaxalone, the ACh-induced current was depressed and the waveform of the ionic current was significantly changed (compare (a) and (b)). In (b), an initial fast inward current was followed by a much slower phase in the waveform of the ionic current. The slow phase was apparent when the tip of the ionophoretic electrode was located near the cell membrane, and the ionophoretic current was realitively large.

Discussion

The results of this study show that the steroid anaesthetic alphaxalone blocks the ACh-induced response in cultured chick myoballs (Fischbach & Lass, 1978), $ED_{50} = 6.0\,\mu M$. At the molecular level, alphaxalone produces an 'all or none' block of the ionic channel and reduces the number of available channels for a given dose of ACh. In double pulse conditioning experiments, alphaxalone induces an additional ('extra') inhibition with a time constant of recovery of 550 ms. The time constant of recovery from normal desensitization is only 250 ms or less.

The blocking action of alphaxalone may be interpreted in terms of two alternative models: (1) enhancement of ACh receptor desensitization; (2) blockade of 'open' channels. The alphaxalone-induced additional inhibition as revealed by the double pulse conditioning experiments favours the second alternative since the time constant of recovery from receptor desensitization is not expected to change due to the effect of the drug (Rang & Ritter, 1970 a, b).

A simple model of channel block by alphaxalone assumes that the steroid anaesthetic blocks active (open) ionic channels like local anaesthetics and other drugs (Adams, 1976; Ruff, 1977; Feltz *et al.*, 1977; Katz & Miledi, 1978; Alder *et al.*, 1978). The kinetic scheme is described by the following three states of equilibrium:

$$\text{closed} \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} \text{open} \underset{b}{\overset{f\,c}{\rightleftharpoons}} \text{blocked} \qquad (1)$$

where α, β, f and b are the rate constants, c is the alphaxalone concentration, and 'closed' is a receptor-ACh complex before the conformational change. Quantitative analysis of the model (Adams, 1976; Adams & Feltz, 1980) shows that:

$$\frac{1}{\tau_f} \simeq \alpha + f\,c \qquad (2)$$

$$\frac{1}{\tau_s} \simeq \frac{1}{b} \qquad (3)$$

Where τ_f is the apparent time the channel is open in the presence of alphaxalone obtained from noise

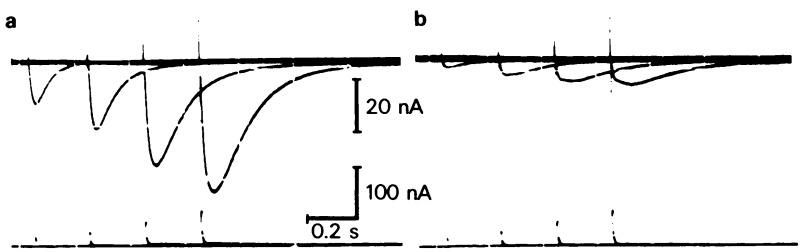

Figure 8 Ionophoretic dose-response of the acetylcholine (ACh)-induced membrane current. ACh was applied every 30 s and the responses were shifted and superimposed (a). In the presence of 60 μM alphaxalone (b) the responses were depressed and a change in the response waveform is evident with a large dose of ACh.

analysis (3.7 ms, see Results) and τ_s is the time constant of decay of the additional inhibition measured in double pulse conditioning experiments (550 ms, see Results).

Equations (2) and (3) are valid when

$$\frac{1}{\tau_f} >> \frac{1}{\tau_s}$$

and at low alphaxalone and ACh concentrations.

Since the apparent channel open time (τ_f) was not affected in approx. 10 μM alphaxalone (c) and the control channel open time $1/\alpha$ is 3.6 ms ($\alpha = 270 s^{-1}$), it can be assumed that the forward rate constant (f, equation 2) in this preparation is $10^6 M^{-1}s^{-1}$ or less (see equation 2).

The f value in embryonic receptors is known to be relatively low compared to that in junctional receptors (Neher & Steinbach, 1978; Ruff, 1982). The value of τ_f derived by measuring the decay of the miniature endplate current (m.e.p.c.) is shortened in the presence of alphaxalone (Torda & Gage, 1977; Pennefather & Quastel, 1980). No such change in the τ_f was found in our study. This discrepancy can be explained by equation 2; the product of f × c must be large compared to α in order to induce a significant reduction in τ (Katz & Miledi, 1978, Table 1). In noise analysis, a steady state concentration of ACh is added and the ED_{50} of a blocking drug is much smaller than when ACh is applied to the nerve (Adams *et al.*, 1970; Albuquerque & Gage, 1978). Indeed, Torda & Gage (1977) employed 220 μM rather than 6–24 μM used in our study, and τ was reduced to only 67% of control. Presumably, the product of f × c (equation 2) in their study was relatively large and τ_f became smaller.

An alternative model for receptor block by a drug involves effects on the receptor desensitization (Rang & Ritter, 1970a, b; Magazanik & Vyskocil, 1973). This model seems unlikely to apply for alphaxalone since the drug produced an 'extra' inhibition with a time constant of recovery larger than the time constant of recovery from normal receptor desensitization. Drugs which affect desensitization do not change this time constant (Magazanik & Vyskocil, 1977; Anwyl & Narahashi, 1980). Also, a significant change in the waveform of the ACh response became apparent when the cell was treated with alphaxalone and the ACh dose was increased (Figure 8). An initial fast response was followed by a much slower surge of current as the activated receptors under the iohophoretic electrode were blocked by the drug (Adams, 1976).

The precise molecular site of alphaxalone interaction with the channel is unknown. Alphaxalone is a large, rigid, hydrophobic molecule and therefore penetration into the channel 'lumen' seems unlikely. The drug may block the channel by allosteric interaction from adjacent hydrophobic sites at the receptor-channel complex or its micro-environment.

In vivo experiments (Richards & White, 1981) with binary mixtures of alphaxalone and other short acting anaesthetic agents showed a greater potency than would be expected if their effects were simply additive. These results were interpreted as evidence against the 'unitary' hypothesis of anaesthesia (Seeman, 1972; Hill, 1974; Smith, 1974; Trudell, 1977) and point to interactions with a specific neuronal membrane structure. The interaction of alphaxalone with open ionic channels as described in this study further support this notion.

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