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Interactions between molecules of a steroid anaesthetic (alphaxalone) and ionic channels of nodal membrane in voltage-clamped myelinated nerve fibre

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1 The effects of the anaesthetic alphaxalone (0.05 to 1 mM) on the node of Ranvier of isolated myelinated nerve fibres of the frog were studied under voltage-clamp conditions.

2 When added to the solution bathing voltage-clamped nodes, alphaxalone modified neither linear leakage nor capacitative currents but rapidly and reversibly blocked K and Na currents. The blocking effects of the anaesthetic on both types of current were not dependent on the frequency of stimulation of the nerve fibres between 0.7 and 10 Hz.

3 The kinetics of the Na current were not modified by alphaxalone but, in the presence of the drug, the K current showed an apparent fast inactivation.

4 Alphaxalone rapidly and reversibly shifted towards negative voltages both the steady-state K conductance-voltage and the peak Na steady-state inactivation-voltage relationships, without noticeable modification of their shape. In contrast, the anaesthetic reversibly decreased the slope of the peak Na conductance-voltage curve.

5 The reduction of the K current induced by alphaxalone was voltage-dependent with an apparent dissociation constant first decreasing from about 0.25 to 0.08 mM between −20 mV and +20 mV and then remaining constant above +20 mV. In contrast, the apparent dissociation constant for the Na current was almost constant with increasing voltages and equalled about 0.30 mM. Hill coefficient values for both K and Na currents were noticeably less than one.

6 It is concluded that, at higher concentrations than those attainable in the brain or in the plasma during surgical anaesthesia in man, alphaxalone has a 'local anaesthetic-like' action on the peripheral nervous system in that it specifically and differentially interacts with K and Na channel gating systems: it is suggested that the anaesthetic would preferentially modify open K and inactivated Na channels.

Introduction

The steroid alphaxalone (3α-hydroxy-5α-pregnane-11,20 dione) (see Figure 1) is the main active component in the anaesthetic Althesin, which has been used clinically to produce a rapid induction of anaesthesia or as principal anaesthetic (Stanley, 1981).

In many classes of anaesthetics, it has been widely assumed as a working hypothesis that anaesthetic activity results from nonspecific interactions with the membrane lipids. This hypothesis derives from the experimental evidence that there is a good correlation between a compound's anaesthetic potency and its oil/water partition coefficient (Seeman, 1972). Interactions between steroid anaesthetics related to, and including, alphaxalone and lipid bilayers have been demonstrated (Connor *et al.*, 1974; Lawrence & Gill, 1975; Makriyannis & Fesik, 1983; Fesik & Makriyannis, 1985). However, minor changes in the structure of alphaxalone with no significant effect on its partitioning properties can lead to large differences in its activities (Connor *et al.*, 1974; Lawrence & Gill, 1975; Makriyannis & Fesik, 1980; Makriyannis & Fesik, 1983; Fesik & Makriyannis, 1985). To account for this structural specificity, two main alternative hypotheses have been proposed. Some

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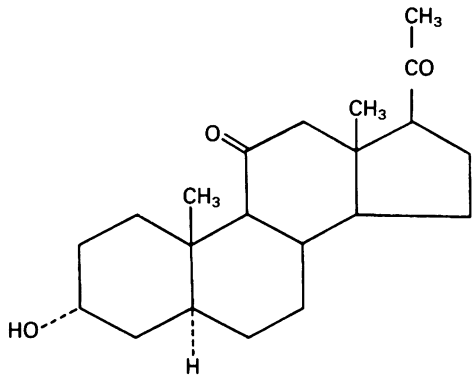

Figure 1 Chemical structure of alphaxalone.

investigators (Lawrence & Gill, 1975; Torda & Gage, 1977; Fesik & Makriyannis, 1985) have hypothesized that the interactions between membrane lipids and alphaxalone molecules are governed by specific structural requirements. This type of interaction would lead to perturbations in the membrane which are sensed by the target proteins. Others (Richards *et al.*, 1978; Pennefather & Quastel, 1980) have suggested that alphaxalone molecules interact directly with the target membrane proteins.

It has been observed that the acetylcholine-induced ionic channels of voltage-clamped myoballs in culture are blocked by high micromolar concentrations of alphaxalone in the open state (Gillo & Lass, 1984). This finding substains the hypothesis of direct interactions between steroid molecules and membrane proteins, i.e., acetylcholine receptors. However, in isolated chromaffin cells, this pharmacological action of alphaxalone does not require a structural specificity (Cottrell *et al.*, 1987). In contrast, several studies have established selective and stereospecific effects of low micromolar concentrations of alphaxalone on γ-aminobutyric acid receptors (Harrison & Simmonds, 1984; Barker *et al.*, 1987; Cottrell *et al.*, 1987; Harrison *et al.*, 1987a; Lambert *et al.*, 1987). These results strongly suggest that, during surgical anaesthesia, one mechanism by which alphaxalone may produce its depressant effects, on the level of the central nervous system, involves enhancement or mimicry of the action of the transmitter γ-aminobutyric acid. This mechanism probably results from direct interactions between the steroid molecules and the transmitter receptors (but see Harrison *et al.*, 1987b).

The aim of the present work was to determine the mode of action of alphaxalone on the peripheral nervous system. We have examined the effects of the steroid anaesthetic on the ionic conductances of the voltage-clamped myelinated nerve fibre. This preparation was used to determine the type of interactions between alphaxalone molecules and ionic channels of nerve membrane. To our knowledge, it is the first time that such investigations, made *in vitro* on this type of excitable membrane, have been described.

Methods

The experiments were carried out on nodes of Ranvier of isolated myelinated nerve fibres from the sciatic nerve of the frog *Rana esculenta*. The nodal membrane was voltage-clamped by the method of Nonner (1969). The normal resting potential of fibres was assumed to be -70 mV, corresponding to 30% of peak Na inactivation (Stämpfli & Hille, 1976). If not stated otherwise, the fibres were stimulated at a frequency of 0.7 Hz. Membrane currents were calculated assuming an axoplasmic resistance of 10 MΩ. Linear leakage and capacitative currents were subtracted electronically from the total current. The series resistance was not compensated for (see Chiu, 1977; Benoit *et al.*, 1985; Dubois & Benoit, 1985).

The Ringer solution had the following composition (mM): NaCl 111.5, KCl 2.5, $CaCl_2$ 1.8, $NaHCO_3$ 2.4, pH 7.4. The fibre ends were cut in a solution containing 120 mM KCl, which was used in the end pools throughout the experiments. When monitoring Na current, K current was suppressed by adding tetraethylammonium (10 mM) to the external solution. The temperature was maintained at about 15°C.

Alphaxalone was provided by Glaxo Group Research Ltd, Greenford, England. The steroid was first prepared as a 50 mM solution in ethanol and then diluted into the Ringer solution before use. The final concentration of ethanol in the Ringer solution did not exceed 2%. Although this amount of alcohol did not affect the currents of the nodal membrane more than 10% in control experiments (see also Århem & Van Helden, 1983), ethanol was also added as appropriate to the Ringer solution under control conditions.

Results

Effects on the time course of K and Na currents

Alphaxalone modified neither linear leakage nor capacitative currents but altered K and Na currents recorded from voltage-clamped nodes of Ranvier.

Figure 2 shows the effects of alphaxalone (0.25 and 1 mM) on the K current. About 15 to 30 s after addi-

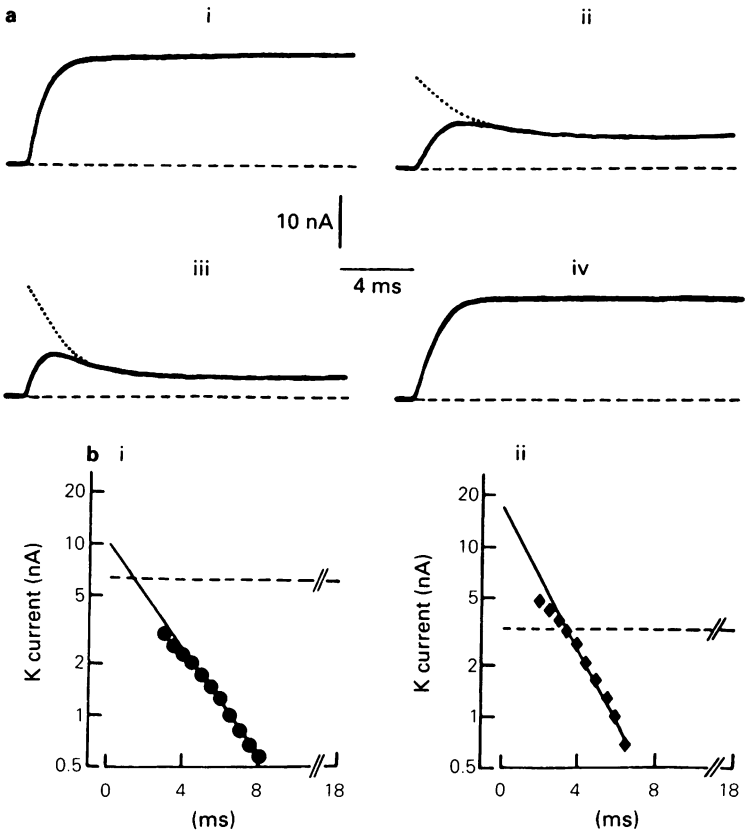

Figure 2 Effects of alphaxalone on the time course of K current. (a) Traces of K current recorded under control conditions (i), 3 min after addition of 0.25 (ii) or 1 mM (iii) alphaxalone to the external solution and 20 min after washout of the drug (iv), during 19 ms depolarizations to +70 mV preceded by 50 ms hyperpolarizations to −120 mV. Under control conditions and in the presence of 0.25 and 1 mM alphaxalone, the steady-state K current was 21.67 nA, 6.33 nA and 3.33 nA, respectively. In (ii) and (iii), dotted curves represent the exponential extrapolation, to time zero of depolarization, of the apparent incomplete inactivation, induced by alphaxalone, whose semilogarithmic representation is shown in (b). (b) (●) and (◆) give the K current, in the presence of 0.25 (i) and 1 mM (ii) alphaxalone, during its inactivation phase minus the steady-state K current linearly extrapolated to time zero of depolarization (broken lines). Extrapolations to time zero of depolarization of the alphaxalone-induced inactivation (straight lines through (●) and (◆)) equalled 10.39 nA (i) and 17.76 nA (ii) and were determined by linear-regression analysis using logarithms of the measured values ($r^2 = 0.99$).

tion of the anaesthetic to the external solution, the K current was reduced and showed an apparent fast incomplete inactivation (Figure 2ai and aiii) which could be described by an exponential function (Figure 2b). The exponential extrapolation to time zero of depolarization of the alphaxalone-induced inactivation added to the steady-state K current, measured in the presence of the drug, gave values between about 80% and 100% of that of the steady-state K current recorded under control conditions. This time-dependent reduction of the K current by alphaxalone was consistently observed in three different fibres in a concentration range of anaesthetic varying from 0.1 to 1 mM. The effects of alphaxalone on the K current were not modified by increasing the frequency of stimulation of the nerve fibres from 0.7 to 10 Hz and were fully reversed by about a 5 to 10 min wash with Ringer solution (Figure 2aiv).

The effects of external alphaxalone (0.5 and 1 mM) on the Na current are shown in Figure 3. The main effect of the anaesthetic was to reduce the peak Na current in about 30 to 60 s without any noticeable change in the current kinetics. Under control conditions, the inactivation time course of Na current can be separated into two exponential phases (Chiu, 1977; Benoit *et al.*, 1985) corresponding to the

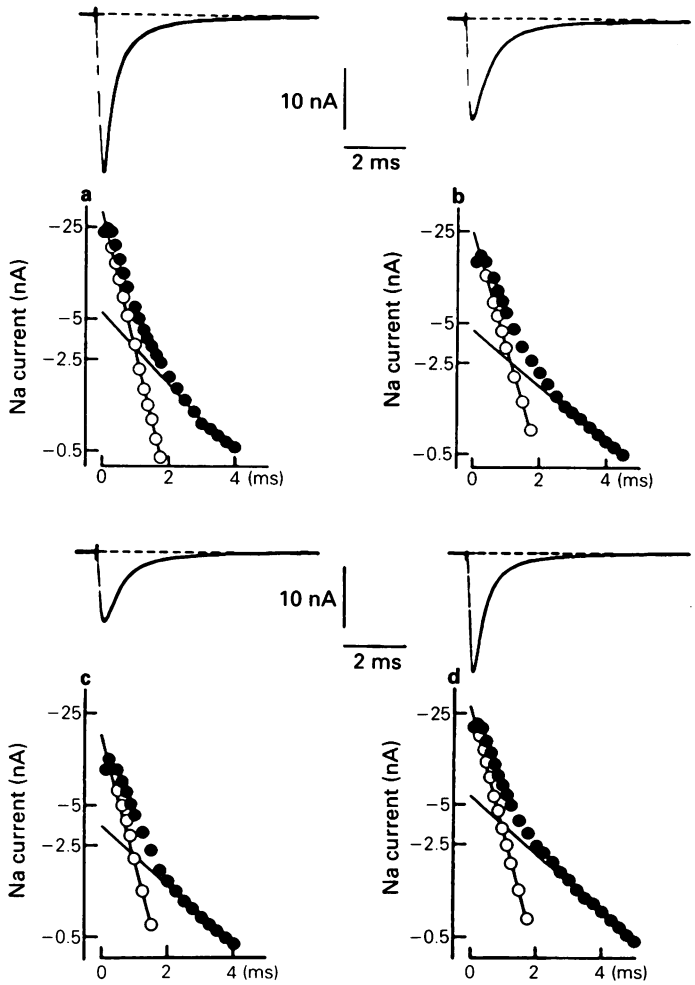

Figure 3 Effects of alphaxalone on the time course of Na current. (a), (b), (c) and (d) Traces of Na current (upper traces) and representations on semilogarithmic coordinates of their inactivation phases (lower panels) under control conditions (a), 5 min after addition of 0.5 (b) or 1 mM (c) alphaxalone to the external medium and 25 min after washout of the drug (d). Na current was recorded during depolarizations to 0 mV preceded by 50 ms hyperpolarizations to −120 mV. In lower panels, (●) give the total current and (○) give the fast phase of inactivation after subtraction of the slow phase of inactivation (straight line through (●)). The values of initial amplitudes to time zero of depolarization and time constants of fast and slow phases of inactivation were determined by linear-regression analysis using logarithms of the measured values ($r^2 \geqslant 0.97$). Time constants of fast and slow phases of inactivation were, respectively, 0.40 ms and 1.65 ms (a), 0.49 ms and 2.01 ms (b), 0.43 ms and 1.91 ms (c) and 0.46 ms and 1.95 ms (d). Initial amplitudes of fast and slow phases of inactivation relative to their values under control conditions were, respectively, 0.75 and 0.78 (b), 0.61 and 0.61 (c) and 0.85 and 1 (d).

straight lines in Figure 3a. In the presence of alphaxalone (Figure 3b and c), the inactivation time course of Na current could still be separated into two exponential phases with almost the same time constants as those under control conditions but with reduced amplitudes. The effects of the anaesthetic on the Na current were apparently not dependent on the frequency of stimulation of the nerve fibres between 0.7 and 10 Hz and were not fully reversed even after 20 to 30 min washing out of the drug with Ringer solution (Figure 3d). After washout of 1 mM alphaxalone, the peak Na current recovered 77.3 ± 1.7% of its control value (mean ± s.e. mean of 4 experiments). When lower concentrations of anaesthetic were used, the reversibility of the effects on the Na current was complete.

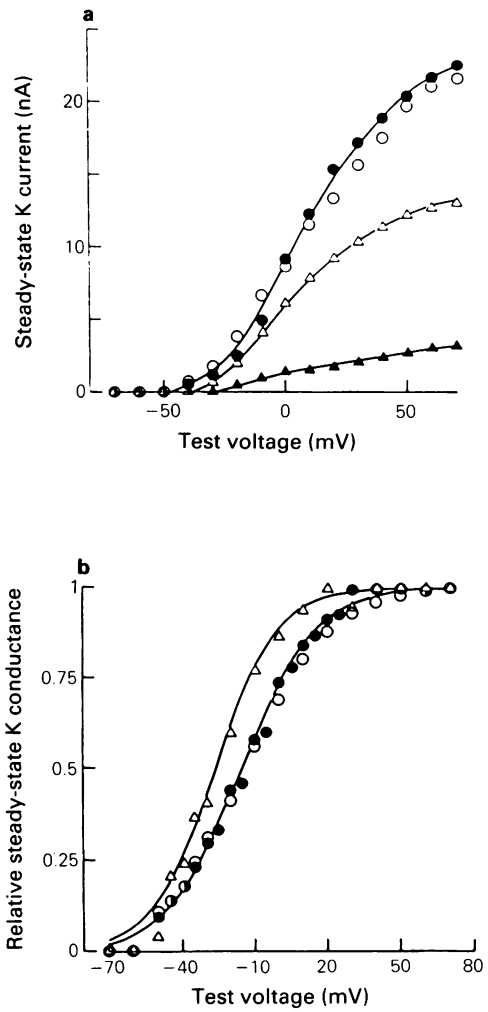

Figure 4 Effects of alphaxalone on the voltage-dependence of the activation of K current. (a) Steady-state K current-voltage relationship and (b) steady-state K conductance-voltage relationship. The K current was measured at the end of either 19 ms (a) or 35 ms (b) depolarizations of various amplitudes preceded by 50 ms hyperpolarizations to −120 mV, under control conditions (●), in the presence of 0.05 (△) or 1 mM (▲) alphaxalone and after washout of the drug (○). In (b), conductances (g) were calculated from Equation 1 and are expressed relative to the respective maximum conductance at large depolarizations (g_{max}). In (a), the curves were drawn by eye and in (b), the curves were calculated from the equation: $g/g_{max} = 1/[1 + \exp(\bar{V} - V)/k]$, where V is the test voltage, $\bar{V}$ is the test voltage for $g/g_{max} = 0.5$ and k is the steepness factor. The values of the parameters $\bar{V}$ and k, determined by non-linear least square fits of data points ($r^2 \geq 0.98$), were, respectively, −16 mV and 15.3 mV (●) and −27 mV and 12.9 mV (△). Different fibres in (a) and (b).

Effects on the voltage-dependence of the activation of K and Na currents

Figure 4a shows the effects of external alphaxalone (0.05 and 1 mM) on the steady-state K current-voltage relationship. For each test voltage, the steady-state K current was reversibly reduced by the drug. Moreover, it is interesting to note that the reduction of the steady-state K current induced by either 0.05 or 1 mM alphaxalone appeared to increase with increasing test voltage. This latter point will be further analysed (see Figures 7 and 9). The effects of the anaesthetic on the voltage-dependence of the K current activation were further investigated on the steady-state K conductance-voltage curve (Figure 4b). Conductances were calculated from steady-state K current-voltage curves in the absence and in the presence of 0.05 mM alphaxalone, according to the equation:

$$g = I/(V - V_{eq}) \quad (1)$$

where g is the conductance, I is the current amplitude, V is the test voltage and V_{eq} is the equilibrium potential of ions. Under our experimental conditions (see Methods), the equilibrium potential of K ions, calculated from the Nernst equation, was −96 mV. In both media, conductances were normalized to their respective values at large depolarizations. In the presence of 0.05 mM alphaxalone, the steady-state K conductance-voltage curve was shifted towards negative voltages. In a concentration range of anaesthetic varying from 0.05 to 1 mM, the negative shift induced by the drug was constant and equalled about 10 mV (Figure 4b and Table1). Alphaxalone did not significantly modify the shape of the curve (see Figure 4b and Table 1). All the effects of the drug on the steady-state K conductance-voltage curve were reversed after about 5 to 10 min washing with alphaxalone-free solution (Figure 4b and Table 1).

The effects of external alphaxalone (0.1 and 1 mM) on the peak Na current-voltage curve are shown in Figure 5a. For each test voltage, the peak Na current was decreased by the drug without noticeable modification in its voltage-dependence, i.e., the voltage at which the peak Na current activated and its reversal potential were almost constant and equalled −50 mV and +80 mV, respectively. In contrast with the steady-state K current (see Figure 4a), the reduction of the peak Na current induced by either 0.1 or 1 mM alphaxalone did not appear to be voltage-dependent (see also Figures 8 and 9). As already observed in Figure 3, the effects of the anaesthetic on the peak Na current were not fully reversed after washing out 1 mM alphaxalone with Ringer solution. Figure 5b presents the action of alphaxalone on the peak Na conductance-voltage relationship.

Table 1 Effects of alphaxalone on voltage characteristics of K conductance

	$\bar{V}$ (mV)	$\Delta\bar{V}$ (mV)	k (mV)	*Relative k*
Control conditions	-8.50 ± 3.39 ($n = 3$)	0.00	14.29 ± 2.48 ($n = 3$)	1.00
Alphaxalone 0.05 mM	-18.88 ± 5.57 ($n = 2$)	-10.38	11.73 ± 2.57 ($n = 2$)	0.82
Alphaxalone 0.25 mM	-20.20 ± 6.96 ($n = 2$)	-11.70	9.79 ± 0.11 ($n = 2$)	0.69
Alphaxalone 1 mM	-20.92 ± 4.73 ($n = 3$)	-12.42	12.96 ± 1.52 ($n = 3$)	0.91
After washout	-8.87 ± 5.11 ($n = 2$)	-0.37	14.90 ± 2.44 ($n = 2$)	1.04

$\bar{V}$ is the test voltage corresponding to half maximum steady-state K conductance.
$\Delta\bar{V}$ is the value of $\bar{V}$ minus that under control conditions.
k is the steepness factor of the steady-state K conductance-voltage curve.
Relative k is the value of k relative to that under control conditions.
The values of the parameters $\bar{V}$ and k were determined by non-linear least square fits of data points ($r^2 \geqslant 0.98$).
Mean $\pm$ s.e. mean of n determinations. (See legend of Figure 4b for more details).

Conductances were calculated from peak Na current-voltage curves in the absence and in the presence of 1 mM alphaxalone, according to Equation 1 and were normalized to their respective values at large depolarizations. In Equation 1, the equilibrium potential of Na ions (V_{eq}) was the reversal potential of the peak Na current. The main effect of the anaesthetic was to decrease the slope of the peak Na conductance-voltage curve. In consequence, the test voltage corresponding to half maximum peak Na conductance (0.5) was more positive in the presence of alphaxalone than under control conditions. The reduction of the slope of the curve was dependent on the anaesthetic concentration and for a concentration range of alphaxalone varying from 0.1 to 1 mM, the slope of the curve was decreased by about 20% and 50% of its control value, respectively (Table 2). This effect of the drug on the peak Na conductance-voltage relationship was fully reversed by about a 5 to 10 min wash with Ringer solution (see Figure 5b

Table 2 Effects of alphaxalone on voltage characteristics of Na conductance and inactivation

	Na conductance				*Na inactivation*			
	$\bar{V}$ (mV)	$\Delta\bar{V}$ (mV)	k (mV)	*Relative k*	$\bar{V}$ (mV)	$\Delta\bar{V}$ (mV)	k (mV)	*Relative k*
Control conditions	-35.50 ± 3.00 ($n = 4$)	0.00	2.83 ± 0.59 ($n = 4$)	1.00	-64.62 ± 0.62 ($n = 4$)	0.00	7.44 ± 0.29 ($n = 4$)	1.00
Alphaxalone 0.1 mM	-35.56 ± 3.77 ($n = 3$)	-0.06	3.45 ± 0.80 ($n = 3$)	1.22	-74.87 ± 4.99 ($n = 2$)	-10.25	7.75 ± 1.03 ($n = 2$)	1.04
Alphaxalone 0.5 mM	-32.42 ± 3.83 ($n = 3$)	3.08	4.07 ± 0.56 ($n = 3$)	1.44	-70.17 ± 0.83 ($n = 2$)	-5.55	8.04 ± 1.20 ($n = 2$)	1.08
Alphaxalone 1 mM	-31.72 ± 8.28 ($n = 2$)	3.78	5.56 ± 0.56 ($n = 2$)	1.96	-71.67 ± 0.20 ($n = 2$)	-7.05	7.23 ± 0.64 ($n = 2$)	0.97
After washout	-36.90 ± 1.96 ($n = 3$)	-1.40	2.62 ± 0.82 ($n = 3$)	0.93	-65.04 ± 0.80 ($n = 3$)	-0.42	7.90 ± 1.00 ($n = 3$)	1.06

$\bar{V}$ is the test voltage or the prepulse voltage corresponding to half maximum peak Na conductance or steady-state inactivation, respectively.
$\Delta\bar{V}$ is the value of $\bar{V}$ minus that under control conditions.
k is the steepness factor of the peak Na conductance-voltage curve or of the steady-state inactivation-voltage curve of the peak Na current.
Relative k is the value of k relative to that under control conditions.
The values of the parameters $\bar{V}$ and k were determined by non-linear least square fits of data points ($r^2 \geqslant 0.94$).
Mean $\pm$ s.e. mean of n determinations. (See legends of Figures 5b and 6 for more details.)

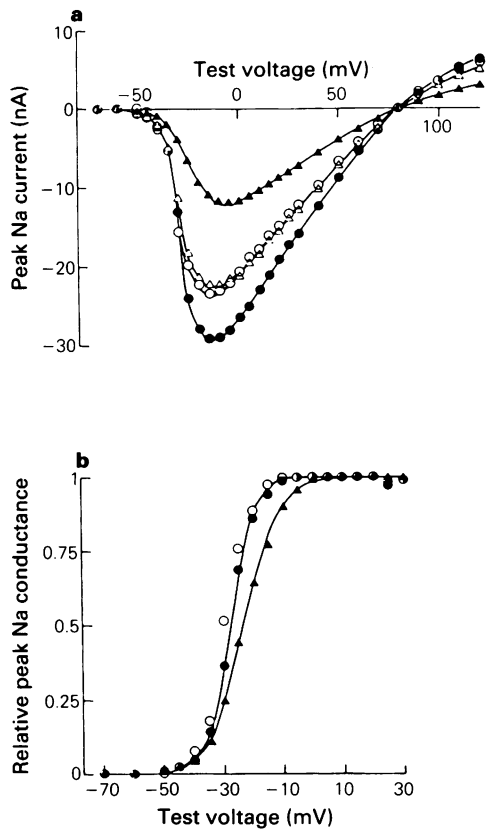

Figure 5 Effects of alphaxalone on the voltage-dependence of the activation of Na current. (a) Peak Na current-voltage relationship and (b) peak Na conductance-voltage relationship. Na current was recorded under control conditions (●), in the presence of 0.1 (△) or 1 mM (▲) alphaxalone and after washout of the drug (○), during depolarizations of various amplitudes preceded by 50 ms hyperpolarizations to −120 mV. In (a), the curves were drawn by eye. In (b), conductances and curves were calculated as described in Figure 4b. The values of the parameters $\bar{V}$ and k ($r^2 \geqslant 0.97$) were, respectively, −28 mV and 4.1 mV (●) and −23 mV and 6.1 mV (▲).

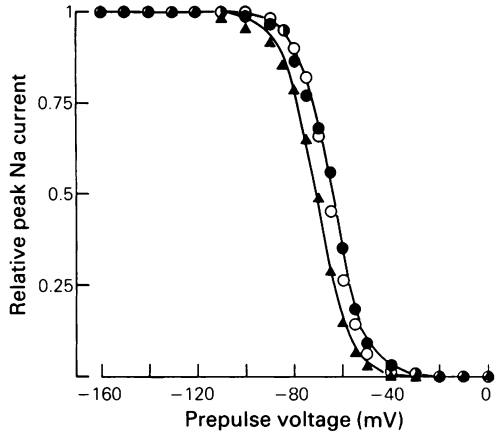

Figure 6 Effects of alphaxalone on the steady-state inactivation-voltage curve of Na current. Na current was recorded under control conditions (●), in the presence of 0.5 mM alphaxalone (▲) and after washout of the drug (○), during depolarizations of 0 mV preceded by 50 ms pulses of various amplitudes. Peak Na current (I) was normalized to its respective value at large hyperpolarizations (I_{max}) and plotted against prepulse voltage (V). The curves were calculated from the equation: $I/I_{max} = 1/[1 + \exp(V - \bar{V})/k]$, where $\bar{V}$ is the prepulse voltage for $I/I_{max} = 0.5$ and k is the steepness factor. The values of the parameters $\bar{V}$ and k, determined by non-linear least square fits of data points ($r^2 \geqslant 0.98$), were, respectively, −64 mV and 7.0 mV (●) and −71 mV and 6.8 mV (▲).

and Table 2), in contrast to the blocking effects of 1 mM alphaxalone on the Na current (see Figures 3 and 5a).

Effects on the steady-state inactivation-voltage curve of Na current

The steady-state inactivation of Na current was studied with the classical two-pulse protocol (Figure 6). In the presence of alphaxalone, the steady-state inactivation-voltage curve of the peak Na current was shifted towards more negative voltages without any significant change in its shape (see also Table 2). This effect of alphaxalone on the steady-state inactivation-voltage curve of the peak Na current occurred within about 1 min of application and was completely reversed after about 5 min washing with an anaesthetic-free solution (Figure 6 and Table 2). It can be noted that further experiments would be needed to determine more precisely the relation between the value of the shift induced by the drug and alphaxalone concentration (Table 2).

Dose-response curve of the effects of alphaxalone on K and Na currents

Figure 7a shows dose-response curves of the steady-state inhibition of K current by alphaxalone (0.05 to 1 mM). In order to see if the reduction of the K current induced by the anaesthetic was voltage-dependent (see Figure 4a), the current was recorded in the absence and in the presence of various concentrations of alphaxalone during depolarizations to −20 mV, 0 mV and +20 mV. The steady-state K current measured at the end of depolarizations was normalized with respect to its corresponding value before the application of the drug. If one assumed

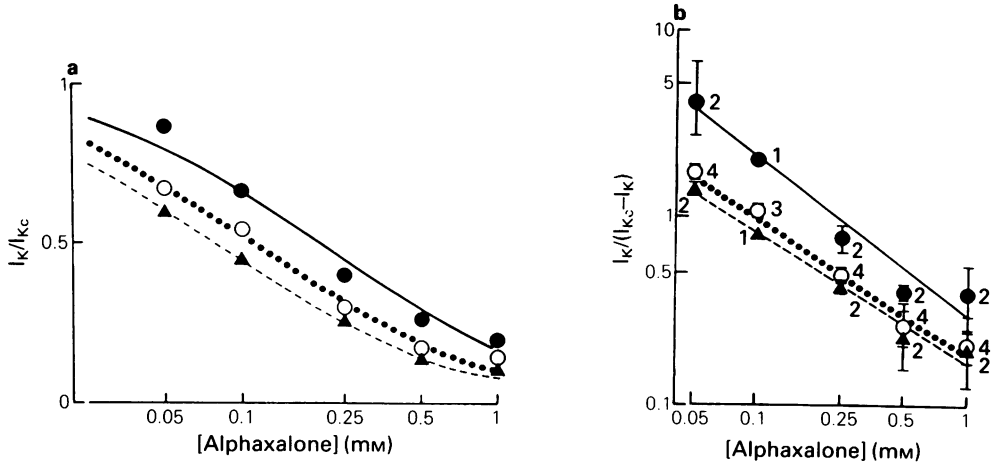

Figure 7 Alphaxalone dose-response relationships for K current. The steady-state K current was recorded at the end of 19 to 35 ms depolarizations to −20 mV (●), 0 mV (○) and +20 mV (▲) preceded by 50 ms hyperpolarizations to −120 mV, in the absence (I_{Kc}) and in the presence (I_K) of 0.05 to 1 mM alphaxalone. (a) Alphaxalone log dose-response curves for the steady-state K current. The steady-state K current was normalized to its value in the absence of drug. The continuous, dotted and dashed curves represent the alphaxalone-induced block of the steady-state K current recorded at −20 mV, 0 mV and +20 mV, respectively, and were calculated from the equation: $I/I_c = 1 - [1/(1 + K/D)]$, where I and I_c are currents, respectively, with and without various concentrations (D) of alphaxalone and K is the apparent dissociation constant. The values of the parameter K, determined by linear-regression analyses ($r^2 \geqslant 0.96$), were 0.22 mM (continuous curve), 0.11 mM (dotted curve) and 0.08 mM (dashed curve). (b) Representations on double logarithmic coordinates (Hill plots) of the effects of alphaxalone on the steady-state K current recorded at −20 mV (continuous line), 0 mV (dotted line) and +20 mV (dashed line) according to the equation:

$$\log (I/(I_c - I)) = \log (K^{n_H}) - n_H \log (D),$$

where I, I_c, K and D are the same parameters described in (a) and n_H is the Hill coefficient. Mean values and standard errors of the mean obtained in 1–4 experiments (numbers besides the points). The straight lines were obtained from linear-regression analyses ($r^2 \geqslant 0.90$) through the points. The values of the parameters K and n_H were, respectively, 0.24 mM and 0.85 (continuous line), 0.10 mM and 0.75 (dotted line) and 0.08 mM and 0.69 (dashed line).

that the inhibition of the steady-state K current by the anaesthetic is described on the basis of a one-to-one reaction between K channels and alphaxalone molecules, the apparent dissociation constant for the steady-state K current increased from 0.08 to 0.22 mM as the test voltage was more negative. The voltage-dependence of the stoichiometric coefficient of the interaction between K channels and alphaxalone molecules (Hill coefficient) is presented in Figure 7b. This Figure shows plots in double logarithmic coordinates (Hill plots) of the experimental data presented in Figure 7a and those obtained from one to three other experiments. The results confirm the increase in apparent dissociation constant for the steady-state K current with decreasing test voltage. Moreover, the Hill coefficient for the steady-state K current, determined from the slope of straight lines, also increased from 0.69 to 0.85 as the test voltage was more negative. It should be noted that Hill coefficient values were noticeably less than one.

Dose-response curves of the blocking effects of alphaxalone (0.05 to 1 mM) on Na current are presented in Figure 8a. In contrast with the blocking effects of alphaxalone on the steady-state K current (see Figure 7a) and as already observed (see Figure 5a), the reduction of the peak Na current induced by the anaesthetic, assuming a one-to-one reaction between Na channels and alphaxalone molecules, did not appear to be voltage-dependent. The apparent dissociation constant for the peak Na current was 0.23 mM. Figure 8b shows a Hill plot of the experimental data presented in Figure 8a and those obtained from one to two other experiments in which Na current was recorded during depolarizations to −20 mV. A Hill coefficient of 0.73, noticeably less than one, was calculated for the peak Na current.

In order to investigate further the voltage-dependence of the blocking effects of alphaxalone, we did representations on semilogarithmic and on normal coordinates of the variations of, respectively,

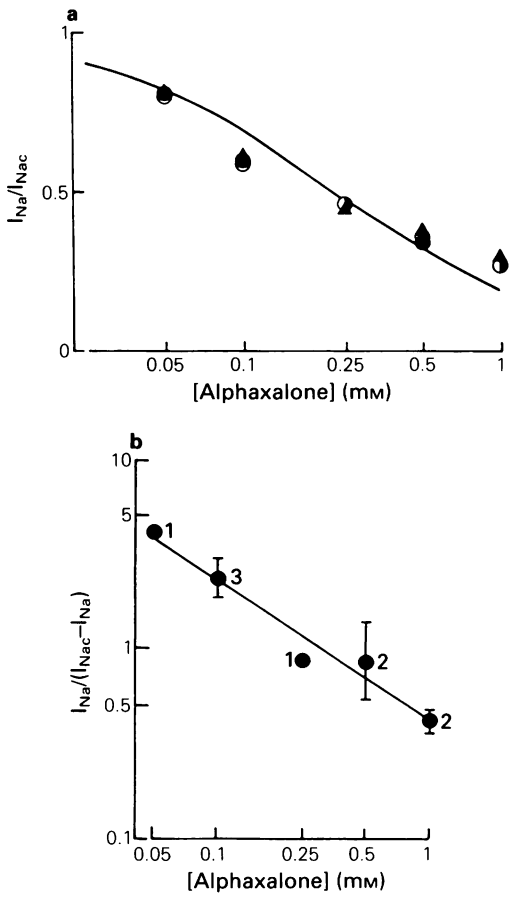

Figure 8 Alphaxalone dose-response relationships for Na current. The peak Na current was recorded during depolarizations to −20 mV (●), 0 mV (○) and +20 mV (▲) preceded by 50 ms hyperpolarizations to −120 mV, in the absence (I_{Nac}) and in the presence (I_{Na}) of 0.05 to 1 mM alphaxalone. (a) Alphaxalone log dose-response curve for the peak Na current. The peak Na current was normalized to its value in the absence of drug. The curve was calculated as described in Figure 7a. The value of the parameter K ($r^2 = 0.96$) was 0.23 mM. (b) Representation on double logarithmic coordinates of the effects of alphaxalone on the peak Na current. The points and the straight line were calculated as described in Figure 7b. Mean values and standard errors of the mean obtained in 1–3 experiments (numbers besides the points). The values of the parameters K and n_H ($r^2 = 0.96$) were 0.31 mM and 0.73, respectively.

the apparent dissociation constant (K) and the Hill coefficient (n_H) with test voltage. In 2–4 experiments, the results obtained for both steady-state K and peak Na currents are presented in Figure 9. Whereas the apparent dissociation constant for the peak Na current was almost constant with increasing test voltage and equalled about 0.30 mM, that for the steady-state K current first decreased from about 0.25 to 0.08 mM between −20 mV and +20 mV and then remained constant above +20 mV (Figure 9a). The Hill coefficient for the peak Na current was also

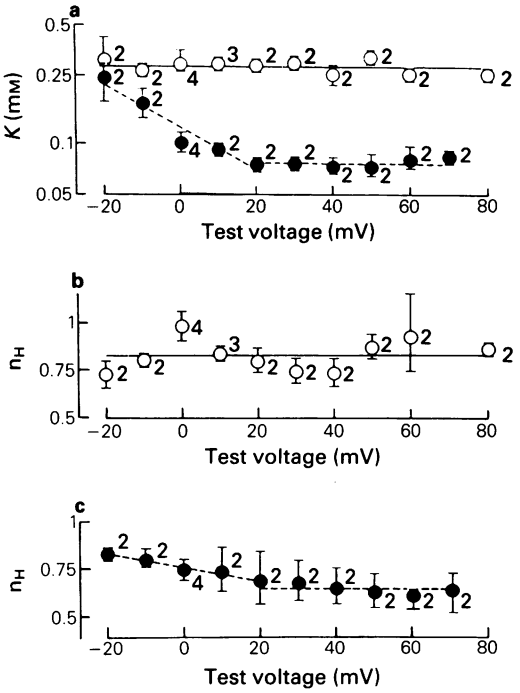

Figure 9 Voltage-dependence of the blocking effects of alphaxalone on K and Na currents. (a) Representations on semilogarithmic coordinates of apparent dissociation constant-voltage relationships for peak Na (○) and steady-state K (●) currents. (b) and (c) Representations on normal coordinates of Hill coefficient-voltage relationships for peak Na (b) and steady-state K (c) currents. Apparent dissociation constants (K) and Hill coefficients (n_H) were calculated as described in Figures 7b and 8b ($r^2 \geqslant 0.90$). Mean values and standard errors of the mean obtained in 2–4 experiments (numbers besides the points). In (a), (b) and (c), dashed lines drawn between +20 mV and +70 mV and the continuous lines are the mean values of the points. Mean values thus determined and standard errors of the mean (number of points) were 0.279 mM ± 0.008 (10) and 0.828 ± 0.027 (10) (continuous lines in (a) and (b), respectively) and 0.077 mM ± 0.002 (6) and 0.640 ± 0.012 (6) (dashed lines drawn between +20 mV and +70 mV in (a) and (c), respectively). In (a) and (c), dashed lines drawn between −20 mV and +20 mV were obtained from linear-regression analyses ($r^2 \geqslant 0.94$) using either logarithms of the mean values (a) or mean values (c) (five points for each line). Voltage constants thus calculated were 34.48 mV (a) and 250.00 mV (c).

constant with the test voltage and equalled about 0.80 (Figure 9b). In contrast, the Hill coefficient for the steady-state K current first decreased from about 0.85 to 0.70 between −20 mV and +20 mV and then remained constant above +20 mV (Figure 9c). It should be noted that Hill coefficient values for both steady-state K and peak Na currents were clearly less than one (see Discussion).

Discussion

The present investigation shows that alphaxalone (0.05 to 1 mM) affects both K and Na currents in myelinated axons. The effects consist mainly of a reduction of both types of currents, the K current being more sensitive to the anaesthetic than the Na current. Modifications of the voltage-dependence of both the activation of K and Na currents and the inactivation of Na current are also present. In contrast with the interactions between Na channels and alphaxalone molecules, those between K channels and alphaxalone molecules appear to be affected by the membrane potential. Both types of interactions are poorly described on the basis of a first order reaction.

Effects on K current

Alphaxalone has two major effects on K current.

The first is a reduction of the K current depending on its time course (see Figure 2). In this respect, the mode of action of alphaxalone on K current is similar to that of several other substances including derivatives of tetraethylammonium (Armstrong & Hille, 1972), quaternary derivatives of local anaesthetics (Strichartz, 1973), strychnine (Shapiro, 1977), quinidine (Revenko *et al.*, 1982), capsaicin (Dubois, 1982), ajmaline (Khodorov & Zaborovskaya, 1983) and the general anaesthetic etomidate (Benoit *et al.*, 1987). The fact that the blocking effect induced on the K current by external application of alphaxalone is rapidly and fully reversed upon washing (see Figures 2 and 4a) may suggest that the receptor site for alphaxalone associated with K channels is located at the external face of the membrane. Its affinity (or accessibility) should then be greater when K channels are in the open rather than in the resting state. Since the probability of K channel opening increases with membrane depolarization and is maximal above about +20 mV (see Figure 4b), this suggestion could explain why the apparent dissociation constant for the K current decreases between −20 mV and +20 mV and remains constant above +20 mV (see Figures 7 and 9a). Another possible explanation for these results would be an increase in the affinity of the receptor site for alphaxalone with membrane depolarization. But, according to this second hypothesis, the decrease of the apparent dissociation constant for the K current should be continuous with increasing test voltage and, in particular, the apparent dissociation constant should not remain constant above +20 mV. However, in the frog node of Ranvier, the interpretation of results can be biased by K accumulation in the nodal gap (Dubois, 1981a; 1983) and by the existence of several components of the K current which have different voltage-sensitivities and pharmacological properties (Dubois, 1981b; 1983; Benoit & Dubois, 1986). Furthermore, the Hill coefficient for the K current also decreases between −20 mV and +20 mV and remains constant above +20 mV (see Figures 7b and 9c). This observation and the fact that Hill coefficient values are noticeably less than one could be explained by assuming either a negative cooperativity of the interactions between the receptor sites for alphaxalone associated with K channels and anaesthetic molecules or alphaxalone having differential effects on the various components of the K current. Further experiments are needed to examine these alternatives more precisely.

The second effect of alphaxalone is to shift reversibly the steady-state K conductance-voltage curve towards more negative values without noticeable change in its shape (Figure 4b and Table 1). This result is to be expected if K channels are preferentially blocked by the anaesthetic in the open rather than in the resting state (see Hille, 1977).

Effects on Na current

Na current is affected by alphaxalone in three ways.

First, the Na current is reduced by the anaesthetic without significant modification in its inactivation kinetics (see Figure 3) with an apparent dissociation constant of about 0.30 mM (see Figures 8 and 9a). As shown for the general anaesthetic etomidate (Benoit *et al.*, 1987), alphaxalone appears to be more efficient in blocking K than Na currents (Figure 9a). This feature of alphaxalone action is in contrast with local anaesthetics and most general anaesthetics which instead preferentially reduce Na rather than K currents (Århem & Frankenhaeuser, 1974; Århem & Rydqvist, 1986). The Hill coefficient calculated for the interactions between Na channels and alphaxalone molecules is about 0.80 (see Figures 8b and 9b). Such a value, clearly less than one, can be explained if one assumes a negative cooperativity of the binding of anaesthetic molecules to their receptor sites associated with the Na channels. However, in the frog node of Ranvier, it has been suggested that the fast and slow phases of Na current inactivation (see Figure 3) correspond to currents flowing through distinct types of Na channels which have

different voltage-sensitivities and pharmacological properties (Benoit *et al.*, 1985). If alphaxalone differentially affected the two components of the Na current, it could explain the calculated value of the Hill coefficient but, according to the results presented in Figure 3, the anaesthetic does not appear to differentiate between the two phases of Na current inactivation.

Secondly, the slope of the peak Na conductance-voltage curve is decreased by alphaxalone (see Figure 5b and Table 2). Assuming that, in the frog node of Ranvier, the series resistance is negligible (see Methods), this would imply that the anaesthetic modifies the activation of the Na current by decreasing the voltage-sensitivity of the gating particles involved in the opening of Na channels (see Haydon & Urban, 1983a,b).

Finally, alphaxalone shifts the steady-state inactivation-voltage curve of the peak Na current towards negative voltages without any significant change in its shape (see Figure 6 and Table 2). According to the modulated receptor model for the action of local anaesthetics proposed by Hille (1977), this result can be interpreted by the assumption that alphaxalone preferentially binds to Na channels which are in the inactivated state. Therefore, the probability of Na channels being in the inactivated state would be increased in the presence of alphaxalone which, in effect, is equivalent to a negative shift of the voltage dependence of the steady-state inactivation of the peak Na current.

We conclude that, in the peripheral nervous system, alphaxalone has a 'local anaesthetic-like' action. However, in contrast with local anaesthetics, alphaxalone may bind preferentially to open K channels rather than to inactivated Na channels. In the peripheral nerve fibres, the steroid anaesthetic activity may be due, at least in part, to interactions between lipids surrounding the channels and alphaxalone molecules. These lipids would then be modified from a crystalline to a fluid phase, allowing the channels to close. According to a membrane: buffer partition coefficient of about 1000 for alphaxalone (Richards & White, 1981), the likely concentration of the steroid in the membrane would be similar to that added in the external solution. A comparison of blocking activity with membrane: buffer partition coefficient yields data which deviate in the blocking potencies of alphaxalone in that the blocking concentration is lower than expected from the partition coefficient (see Figures 7a and 8a). Thus, the present results strongly suggest that alphaxalone alters the membrane permeabilities by specifically and differentially interacting with K and Na channel gating systems. Whereas many drugs are considered 'local anaesthetics' by their impulse blocking capability, there are probably several different modes and, perhaps, different receptor sites by which this result is effected.

Several pieces of evidence indicate the likely relevance of alphaxalone actions on γ-aminobutyric acid receptors in mediating the steroid depressant effects on the level of the central nervous system (see references in Introduction). In particular, the anaesthetic concentrations used in these studies are in the range of those measured in the plasma during surgical anaesthesia in man, i.e., 1 to 10 μM (Sear & Prys-Roberts, 1979). In the present research, alphaxalone concentrations (0.05 to 1 mM), 50 to 100 times higher than those needed to obtain anaesthesia, were active. At this purpose, we must point out that, although extrapolation of our results to man can be invalidated in view of the importance of species differences, peripheral effects of alphaxalone may have some significance when the compound is used with a continuous infusion as complete anaesthetic technique.

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