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POTENTIATION OF γ-AMINOBUTYRIC-ACID-ACTIVATED CHLORIDE CONDUCTANCE BY A STEROID ANAESTHETIC IN CULTURED RAT SPINAL NEURONES

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SUMMARY

1. Intracellular recordings from cultured rat spinal cord neurones demonstrated that Cl^--dependent responses to GABA (γ-aminobutyric acid) (but not glycine) were increased in amplitude and duration by the steroid anaesthetic alphaxalone (3α-hydroxy-5α-pregnane-11,20-dione) at submicromolar concentrations that produced little or no effect on passive electrical properties. The non-anaesthetic 3β-hydroxy analogue was without effect on GABA-evoked responses.

2. Under voltage clamp, membrane currents evoked by GABA were potentiated by alphaxalone without change in the reversal potential for the GABA-evoked response. Fluctuation analysis of GABA-evoked currents suggested that the mean open-time of GABA-activated channels was prolonged from 30 to 74 ms in the presence of the anaesthetic.

3. Higher concentrations of alphaxalone, similar to those reported during surgical anaesthesia, increased membrane conductance in the absence of exogenously applied GABA. Under voltage clamp, current responses to alphaxalone reversed at the same potential as did responses to GABA, suggesting that they result from increased Cl^- conductance.

4. Alphaxalone responses were reduced by the GABA antagonist bicuculline. Fluctuation analysis of current responses to the anaesthetic suggest that they result from the activation of ion channels of long (100 ms) open-time and elementary conductance indistinguishable from that of channels activated by GABA (20 pS). Taken together, these findings indicate that the steroid anaesthetic is able to directly activate Cl^- conductance normally activated by GABA in spinal neurones.

5. The actions of the steroid at GABA-receptor–Cl^--channel complexes are similar to those produced by the anaesthetic barbiturates (e.g. pentobarbitone), although obtained at 50–100-fold lower concentrations. These effects on the inhibitory Cl^--conductance mechanism may be partly responsible for the depressant actions of alphaxalone on the mammalian central nervous system.

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INTRODUCTION

Intravenous administration of certain steroids induces general anaesthesia in animals (Selye, 1941). This initial observation led to the development of steroidal anaesthetics for clinical use (Laubach, P'an & Rudel, 1956; Child, Currie, Davis, Dodds, Pearce & Twissell, 1971). The steroid alphaxalone (3α-hydroxy-5α-pregnane-11,20-dione) is the major active constituent of one such anaesthetic formulation, producing surgical anaesthesia in man at low micromolar plasma concentrations (Sear & Prys-Roberts, 1979). The corresponding 3β-hydroxy isomer is pharmacologically inactive, providing a striking example of anaesthetic stereoselectivity.

One mechanism by which general anaesthetics may produce their depressant effects on the level of central nervous system (C.N.S.) excitability involves enhancement or mimicry of the action of the transmitter γ-aminobutyric acid (GABA). Activation of Cl^- conductance by GABA in central neurones is usually associated with a decrease in the probability of action potential generation as the cell membrane is 'shunted' and often hyperpolarized from rest (Krnjević, 1974). Pentobarbitone and other anaesthetic barbiturates enhance Cl^--dependent responses to GABA recorded in various preparations of C.N.S. tissue (Nicoll, 1975; Barker & Ransom, 1978; Segal & Barker, 1984) at concentrations that are without effects on passive membrane properties (Barker & Ransom, 1978; Schulz & Macdonald, 1981). Fluctuation analyses of GABA-activated Cl^- currents recorded under voltage clamp show that the average open-time of Cl^- channels contributing to the macroscopic response to GABA is substantially prolonged by pentobarbitone (Study & Barker, 1981). In addition, pentobarbitone activates Cl^- conductance in cultured neurones at about fivefold higher concentrations than those producing enhancement of the GABA-activated chloride conductance. These latter concentrations, however, lie within the range of concentrations associated with clinical anaesthesia (Barker & Ransom, 1978; Schulz & Macdonald, 1981).

Recent pharmacological studies have indicated that the steroid alphaxalone may have similar actions. Alphaxalone prolongs the GABA-mediated dorsal root potential associated with presynaptic inhibition in the cat (Lodge & Anis, 1984), prolongs a GABA-mediated inhibitory potential in guinea-pig olfactory cortex (Scholfield, 1980), and selectively enhances GABA-evoked Cl^--dependent responses in rat cuneate nucleus slices (Harrison & Simmonds, 1984). The steroid anaesthetic has no effect on whole-cell Na^+ currents in bovine chromaffin cells at relevant anaesthetic concentrations (Cottrell, Lambert & Peters, 1985).

In the present study we have examined the interactions between alphaxalone and GABA-activated Cl^- conductance in tissue-cultured rat spinal neurones. Some preliminary observations have been reported previously (Harrison, Vicini, Owen & Barker, 1985; Barker, Harrison, Lange, Majewska & Owen, 1986).

METHODS

Electrophysiological recording

Recording with one or two high-resistance (50–60 MΩ) micro-electrodes in current- and voltage-clamp configurations was performed as previously described (Smith, Barker, Smith & Colburn, 1980). Spinal neurones from the embryonic rat were grown in tissue culture for 4–8 weeks by

procedures similar to those previously used to cultivate mouse spinal neurones (Ransom, Neale, Henkart, Bullock & Nelson, 1977). Micro-electrodes were filled with 3 M-KCl, 3 M-potassium acetate or 3 M-CsCl.

Some experiments were performed with the whole-cell recording configuration of the patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981), using a continuous single-electrode voltage-clamp (List EPC-7). After formation of a high-resistance seal (5–50 GΩ) between membrane and pipette, the patch of membrane enclosed by the pipette was disrupted (Fenwick, Marty & Neher, 1982), providing a low-resistance pathway to the interior of the cell. Cell input resistances of 150–500 MΩ were measured during recordings from 10–20 μm diameter spinal cord neurones.

The patch pipettes were filled with a solution containing (in mM): CsCl, 145; $MgCl_2$, 2; EGTA, 1·1; $CaCl_2$, 0·1; HEPES, 5; titrated to pH 7·2 with KOH; sucrose was added to adjust the osmolarity to 310 mosmol/kg. The patch pipettes had resistances of 2–3 MΩ when filled with this solution and placed in the bathing medium. All recordings were made at room temperature (22–24 °C).

Extracellular medium

The bathing solution consisted of (in mM): NaCl, 130; KCl, 5; $CaCl_2$, 4; $MgCl_2$, 4; HEPES, 5; titrated to pH 7·4 with NaOH (325 mosmol/kg). 1 μM-tetrodotoxin was added in some experiments in order to suppress Na^+-dependent action potentials and spontaneous synaptic activity. In order to reduce the extent of delayed outward rectification that dominates the depolarized membrane in these cells, CsCl-filled electrodes were used or 10 mM-tetraethylammonium (TEA) chloride was added to the bathing medium. For current fluctuation analyses, the recording medium contained 2 mM-$CaCl_2$ and 10 mM-$MgCl_2$, in order to reduce Ca^{2+}-dependent release of transmitter (Barker, McBurney & MacDonald, 1982).

Drug applications

Drug-containing solutions were applied to the cell body of the neurone under study by pressure ejection (< 2 lb/in^2) from micropipettes of 1–2 μm tip diameter. All drugs were dissolved directly in the recording medium, except the steroids which were first dissolved in ethanol at a concentration of 20 mM and then diluted as appropriate. The steroids were kindly supplied by Dr K. Child of Glaxo Group Research Ltd., U.K.

Current fluctuation analysis

Currents measured under voltage clamp with either the List EPC-7 or two-electrode clamp were filtered and sampled in the same way. Current responses to agonists were bandpass filtered using a Krohn–Hite filter (3322R) for high-pass filtering and a Frequency Devices 8-pole Butterworth filter (901F) for low-pass filtering (Barker *et al.* 1982). The frequency band most often used was 0·2–200 Hz. Filtered current was then amplified appropriately and digitized using a Data Translation analog-to-digital converter (12 bit, ±5 V range). Digitized data were acquired and stored by a PDP-11/23 computer in non-contiguous records of 2048 points. Signals were sampled at twice the low-pass filter setting. Epochs were constructed later consisting of between four and ten records acquired under the same condition (e.g. base-line or agonist application). A spectral analysis was made of current fluctuations in each epoch using Fourier analysis and spectral density plots generated over the recording band width. Spectra obtained from base-line periods (normally $1/f$ noise) were subtracted from those obtained during agonist-induced currents and subsequent 'difference spectra' were fitted with a Lorentzian function of the form:

$$S(f) = S(0)/[1+(f/f_c)^2],$$

where $S(f)$ is the spectral density, $S(0)$ the zero frequency asymptote, f is frequency and f_c the corner frequency (the frequency at which the spectral density is half-maximum).

Assuming a simple two-state model of elementary channel behaviour, the average channel lifetime (τ) may be calculated from:

$$\tau = \frac{1}{2\pi f_c}$$

and the elementary conductance (γ) obtained from:

$$\gamma = \frac{\text{var}}{I_N V_D},$$

where var is the mean experimental current variance, I_N is the macroscopic current response to agonist and V_D is the driving force for the conducting ion, provided that the probability of an individual ion channel being in the open state is $\ll 1$ (Anderson & Stevens, 1973). At levels of response associated with low concentrations of GABA, previous work on cultured spinal neurones has shown that var $\propto I_N$ (Study & Barker, 1981; Barker *et al.* 1982). This observation provides indirect evidence that individual channels are open rarely at the concentrations of GABA involved in these studies. A more direct method of showing that this is true involves measuring the time spent in the open state by GABA-activated single channels as a percentage of recording time. In outside-out patch recordings from cultured neurones, we estimate individual channels were open approximately 10% of the time in membrane patches held at −60 mV and continuously bathed with a solution containing 1 μM-GABA (J. W. Harrington & N. L. Harrison, unpublished observations). The GABA concentration at the receptor site in the present study would be expected to be in the low micromolar range, when 10 μM-GABA is applied from a micropipette some distance from the cell body, so we feel confident that the assumption of low open probability is justified, at least in the case of control GABA responses. Our conclusions with respect to the potentiating action of the steroid are obviously dependent upon this condition also being met in the presence of the drug.

RESULTS

Alphaxalone potentiates Cl^--dependent responses to GABA

Initial experiments with single KCl-filled electrodes showed that alphaxalone (1 μM in pressure pipette) increased the amplitude of depolarizing responses to brief pulses of GABA in twelve of thirteen cells (Fig. 1*A*). At higher (10 μM) concentrations of alphaxalone, the potentiating effect of the steroid was accompanied by a direct depolarizing action (Fig. 1*B*; see arrow). The potentiating action of the steroid was often associated with a marked increase in the duration of the GABA response (Fig. 1*B*), and reversed fairly rapidly following brief applications.

When prolonged applications of GABA were used to evoke equilibrium responses and membrane resistance simultaneously monitored with brief hyperpolarizing current pulses, alphaxalone was observed to cause a substantial enhancement of both the GABA-evoked depolarization and conductance increase (Fig. 1*C*). Alphaxalone potentiation of GABA responses was also observed in potassium acetate recordings, under which conditions GABA evoked hyperpolarizing responses at the resting membrane potential (Fig. 1*D*).

Alphaxalone did not enhance responses of spinal neurones to the inhibitory amino acid glycine in three cells in which potentiation of GABA was observed, indicating the likelihood of a selective interaction of the anaesthetic with the GABA receptor system. The potentiation of GABA responses by the anaesthetic was a stereoselective phenomenon; experiments in three different cells with the biologically inactive 3β-hydroxy isomer ('betaxalone') demonstrated that this isomer does not potentiate GABA-evoked responses in rat spinal neurones at a concentration of 10 μM. Ethanol (in a final concentration of 0·1%, equal to that present in the highest doses of alphaxalone) was also completely ineffective. Alphaxalone does not appear to enhance responses to GABA by interacting with benzodiazepine receptors. In cultured rat spinal neurones, as previously reported for mouse neurones, the benzodiazepine hypnotic diazepam also potentiates GABA responses. Potentiation of GABA by benzodiazepines was prevented by bath application of the benzodiazepine antagonist Ro15-1788 (25 μM). Addition of Ro15-1788 did not affect the

enhancement of GABA responses by alphaxalone in three cells in which this was tested (not shown).

In rat spinal neurones voltage clamped either with two micro-electrodes (in the case of large neurones) or using the whole-cell patch-clamp method (for smaller cells), membrane currents evoked by GABA were enhanced in amplitude and the GABA-

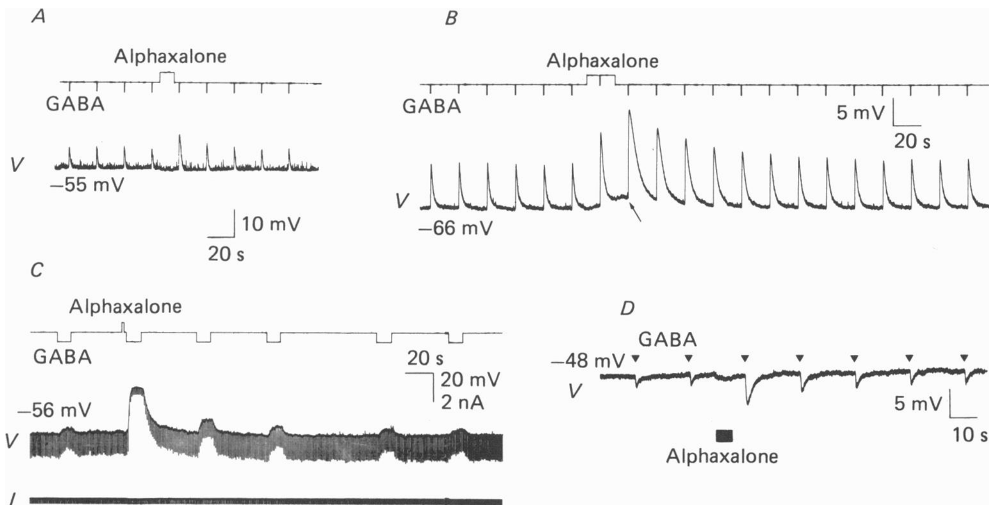

Fig. 1. Alphaxalone enhances voltage responses to GABA. *A*, short (350 ms) pulses of 20 μM-GABA (downward deflexions in upper trace) evoke depolarizing responses from resting potential in this neurone that are increased in peak amplitude by application of 1 μM-alphaxalone from another pressure pipette placed within 10 μm of the cell. *B*, in another cell, application of a higher concentration of alphaxalone (10 μM) increases both amplitude and duration of responses to GABA (20 μM; 150 ms), and in addition causes a small depolarization of the membrane (see arrow). *C*, application of 10 μM-GABA to this cell for 10 s produces a modest depolarization and conductance increase. A brief (2 s) pulse of 10 μM-alphaxalone immediately prior to GABA application results in a striking potentiation of this GABA-evoked response. *D*, potassium acetate recording from a cell held at −48 mV with depolarizing current. 250 ms pulses (arrowheads) of 20 μM-GABA evoke 2–3 mV hyperpolarizing responses that are potentiated by application of 10 μM-alphaxalone. *V*, voltage record; *I*, current record. All recordings with KCl-filled micro-electrodes except in *D*.

evoked conductance increase was augmented (Fig. 2). Alphaxalone did not alter the reversal potential for the GABA-evoked current.

Direct action of alphaxalone on membrane conductance

Current-clamp recordings with either KCl- or potassium-acetate-filled electrodes demonstrated that alphaxalone itself could directly increase membrane conductance. In potassium acetate recordings, both GABA and alphaxalone hyperpolarized the cell, increased membrane conductance and reversibly prevented anode-break spike generation (Fig. 3*A*), while in KCl recordings alphaxalone (10 μM) produced a depolarization from resting membrane potential associated with a conductance increase. The response was similar to that evoked by GABA, but was slower in onset

and offset (Fig. 3*B*). These observations strongly suggest that alphaxalone, like GABA, activates Cl^- conductance, since the polarity of voltage responses to alphaxalone varies with intracellular Cl^- loading in a similar manner to Cl^--dependent responses evoked by GABA.

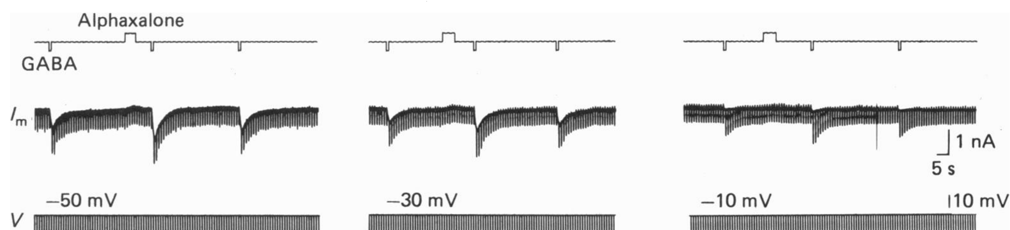

Fig. 2. Alphaxalone potentiates current responses to GABA. Voltage-clamp recording from a rat spinal neurone impaled with two CsCl-filled micro-electrodes. Membrane conductance was periodically assessed from the amplitude of current responses evoked by hyperpolarizing voltage steps (20 mV, 1 Hz, 100 ms). 1 s pulses of 20 μM-GABA were applied to the cell body at various holding potentials. At each holding potential, a short pulse of 1 μM-alphaxalone was applied between GABA applications. The amplitude of GABA-evoked currents is enhanced at −50 and at −30 mV; the reversal potential is unaffected by application of alphaxalone. I_m, membrane current.

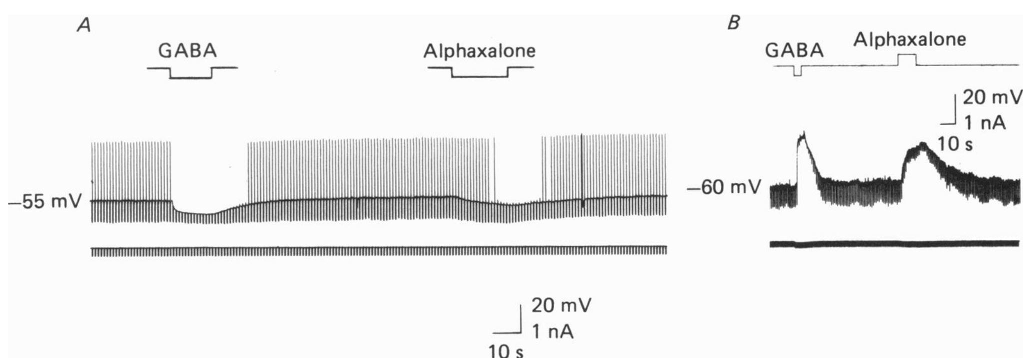

Fig. 3. Alphaxalone directly increases membrane conductance. *A*, potassium acetate recording. Hyperpolarizing current pulses (0·3 nA, 200 ms, 1 Hz) produced anode-break spiking in this neurone, which demonstrated little spontaneous activity. Prolonged pressure application of 20 μM-GABA to the cell body hyperpolarized the membrane by 10 mV and produced an approximate doubling of membrane conductance; anode-break activity was prevented. 10 μM-alphaxalone mimicked the GABA response, except that the onset and decline of the response were slower than was the case for GABA. *B*, KCl recording. GABA (20 μM) and alphaxalone (10 μM) both depolarized this cell and increased membrane conductance.

In whole-cell patch-clamp recordings from small spinal neurones (10–20 μm diameter, 7–14 days in culture), we also observed direct membrane responses to GABA and alphaxalone in six cells. Over recording periods of 1 h, little if any 'wash-out' of responses to low concentrations of GABA was seen in whole-cell recordings. Brief applications of GABA evoked membrane currents that reversed at approximately 0 mV (Fig. 4*A*), and were associated with a substantial increase in membrane conductance (Fig. 4*B*). Under current clamp, depolarizing responses to alphaxalone were recorded at negative membrane potentials; these responses

reversed near 0 mV, and were associated with an increase in membrane conductance (Fig. 4*C*). When the neurones were voltage clamped, alphaxalone-induced membrane currents reversed close to 0 mV (Fig. 4*D*), as was the case for GABA-induced currents

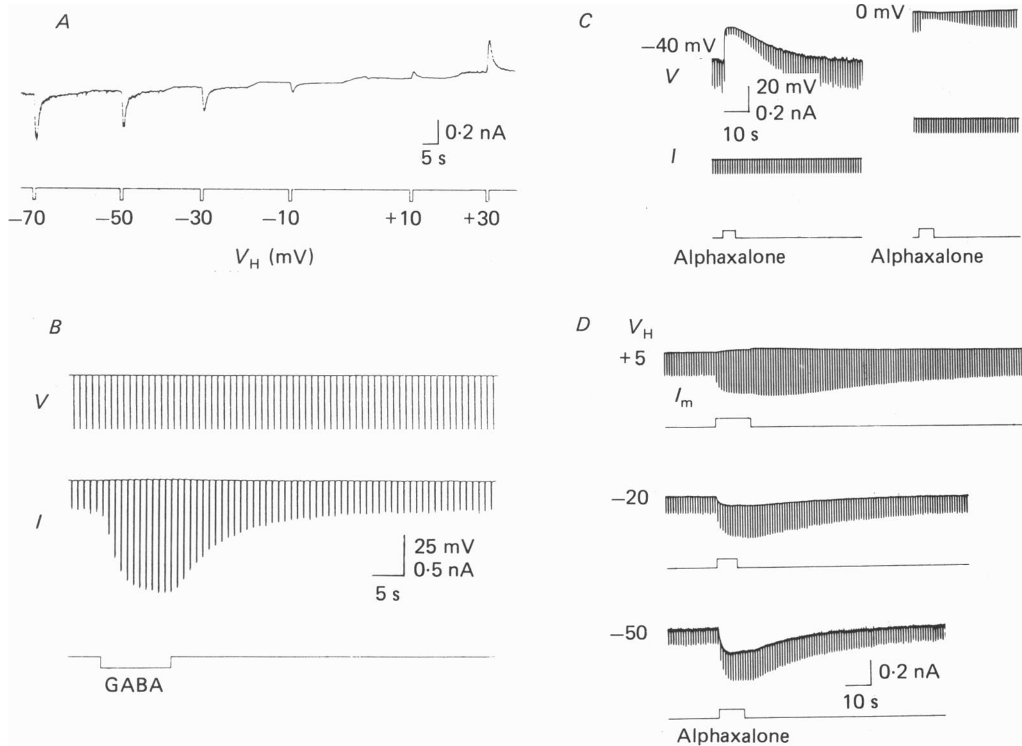

Fig. 4. GABA and alphaxalone evoke membrane currents that reverse near 0 mV. Whole-cell patch-clamp recordings from small diameter rat spinal neurones (10–20 μm diameter, 7–14 days in culture). The intracellular solution contained 145 mM-CsCl. GABA and alphaxalone were applied to the cell body from pipettes containing 10 μM solutions. *A*, current responses to 1 s applications of GABA reverse at approximately 0 mV. In between GABA applications, the holding potential (V_H) was changed to the values indicated below the individual GABA applications. *B*, a prolonged application of GABA at $V_H = +5$ mV elicits little if any net membrane current but produces a large and well-sustained increase in membrane conductance as assessed from the amplitude of current responses to 100 ms, 35 mV voltage jumps. *C*, in current-clamp mode, alphaxalone responses are depolarizing at −40 mV and reverse close to 0 mV. *D*, under voltage clamp, alphaxalone elicits a conductance increase (apparent from the increase in amplitude of current responses to 20 mV hyperpolarizing voltage commands) and membrane currents (I_m) that reverse in polarity close to 0 mV.

under these recording conditions (Fig. 4*A*). These observations further support the idea that alphaxalone may directly activate a Cl^- conductance in rat spinal neurones.

Responses to direct application of alphaxalone were attenuated by the GABA antagonist bicuculline (Fig. 5) and could also be increased in amplitude by diazepam (not shown), consistent with alphaxalone activation of the Cl^--conductance mechanism normally utilized by GABA.

Fluctuation analysis of GABA-evoked current responses potentiated by alphaxalone

Current fluctuation analyses were carried out on spinal cord cells held at −50 mV using either the two-electrode voltage-clamp (large cells) or whole-cell patch-clamp technique (small cells). Between three and fourteen spectra were obtained in each

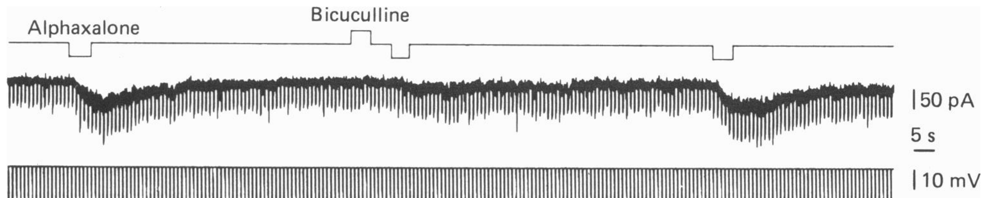

Fig. 5. Bicuculline depresses alphaxalone-evoked responses. CsCl whole-cell recording. In this cell, voltage clamped at −50 mV, a 5 s application of alphaxalone (10 μM) evokes an inward current that is attenuated by prior application of bicuculline (50 μM) to the neurone under study. The response to alphaxalone recovers fully within a minute.

neurone studied. Although there was a clear disparity in cell input resistance between the two techniques, it was possible to evoke stable current responses to GABA, whose power spectra could be described by single Lorentzian functions, irrespective of the recording technique. In two-electrode recordings, larger-amplitude currents were necessarily evoked in order to achieve an acceptable ratio ($>$ 1·5) of drug-induced current variance relative to control. For this reason 20 μM-GABA was applied to cells in two-electrode recordings, while 10 μM-GABA was utilized to evoke smaller amplitude currents in the whole-cell patch-clamp recordings. Power spectra for GABA-evoked currents obtained using the two-electrode voltage-clamp were characteristically more scattered than was the case for spectra recorded with the whole-cell patch technique (Fig. 6), and often revealed an excess of power in the high-frequency end of the spectrum. In contrast, despite the extended recording band width often used in conjunction with the whole-cell patch recording technique, the power spectra of GABA-evoked currents recorded using this latter technique were always well fitted by a single Lorentzian function (Fig. 6). The values of estimated mean open-time (τ) and elementary conductance (γ) obtained using the two techniques were not significantly different. The estimates of τ and γ obtained for GABA alone in this study are summarized in Table 1, and are similar to values previously reported in mouse spinal neurones (Barker *et al.* 1982).

When GABA-evoked currents were increased in amplitude by co-application of alphaxalone (Fig. 7), the power spectra associated with the enhanced current responses were consistently shifted to a lower frequency range (Fig. 8). The spectra were well described by a single Lorentzian function with the corner frequency shifting to a lower value, corresponding to an increase in the mean open-time of the GABA-activated Cl^- channels from 30 to 74 ms (Table 1). Following these prolonged applications of alphaxalone, the estimates of mean open-time obtained for GABA-evoked current responses remained elevated above control estimates for many minutes.

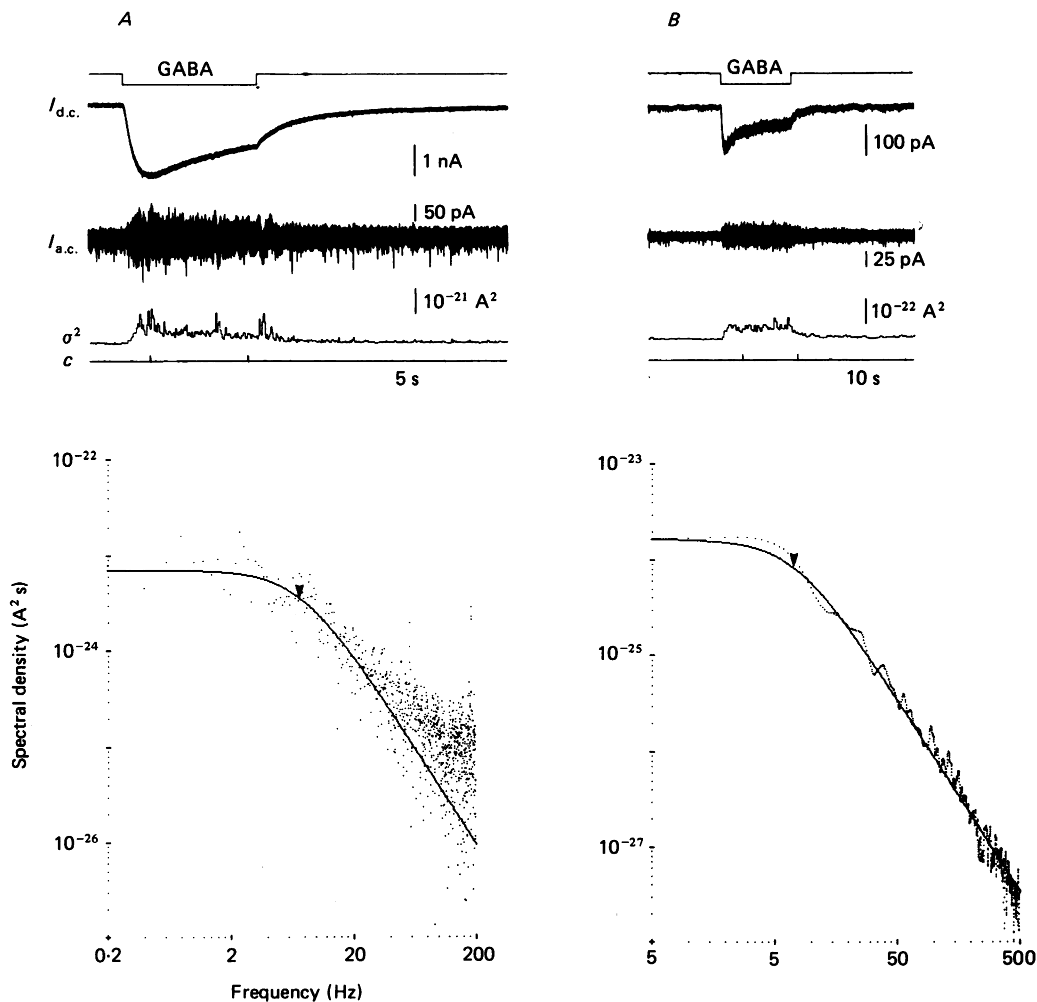

Fig. 6. Macroscopic currents evoked by GABA have similar power spectra recorded under two-electrode voltage clamp or whole-cell patch clamp. *A*, two-electrode voltage-clamp recording of a response to 20 μM-GABA from a rat spinal neurone held at -50 mV, together with the power spectrum for this response, which is fitted with a single Lorentzian function: from the corner frequency (f_c = 7·3 Hz) of this spectrum, one can estimate an apparent mean open-time for the GABA-operated Cl^- channels of 21·7 ms. The elementary conductance of these channels was estimated to be 20 pS. The excess of power seen in the high-frequency range of this spectrum is most likely to reflect imperfect subtraction of noise associated with the voltage clamp used here, since excess power and pronounced scatter in this range is a variable feature of spectra obtained under both control and agonist-activated conditions. *B*, whole-cell patch-clamp recording of a response to 10 μM-GABA in another cell held at -50 mV. The power spectrum could be fitted by a single Lorentzian. Note the much smaller degree of scatter of the data around the computed spectrum relative to the data in *A*. The microscopic parameters were estimated as 22·3 ms (mean open-time) and 15 pS (elementary conductance). $I_{a.c.}$, clamp current with both high- and low-pass filtering. $I_{d.c.}$, clamp current with low-pass filter only; the cut-off frequencies for both high- and low-pass filters are indicated by the minimum and maximum on the frequency axis of the appropriate spectral density plot in this and succeeding figures. σ^2, the output of a device that converts the variance in the filtered a.c. current to a voltage at 0·5 s intervals. *c*, indicates the period of data aquisition by the computer. Arrowheads indicate corner frequency, f_c.

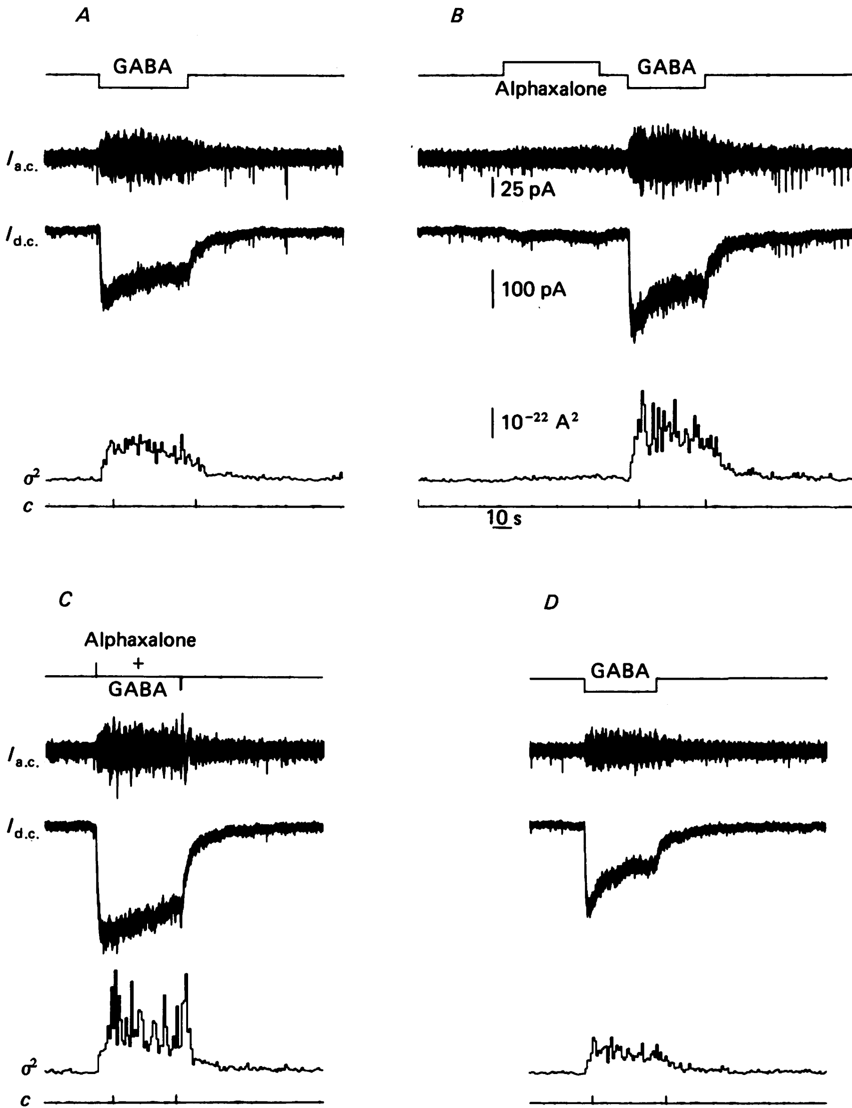

Fig. 7. Alphaxalone increases current responses to prolonged GABA applications. The macroscopic current recorded (whole-cell recording) in response to 10 μM-GABA is enhanced in amplitude by co-application of 1 μM-alphaxalone. The response shown in *A* is a control response to GABA, while the response in *B* was obtained after a prior application of 1 μM-alphaxalone. The response in *C* was obtained during concomitant application of GABA and alphaxalone, while *D* shows a response to GABA obtained approximately 10 min after *C*. The power density spectra calculated for the current responses shown in *A* and *C* are illustrated in Fig. 8. For key to symbols see legend of Fig. 6.

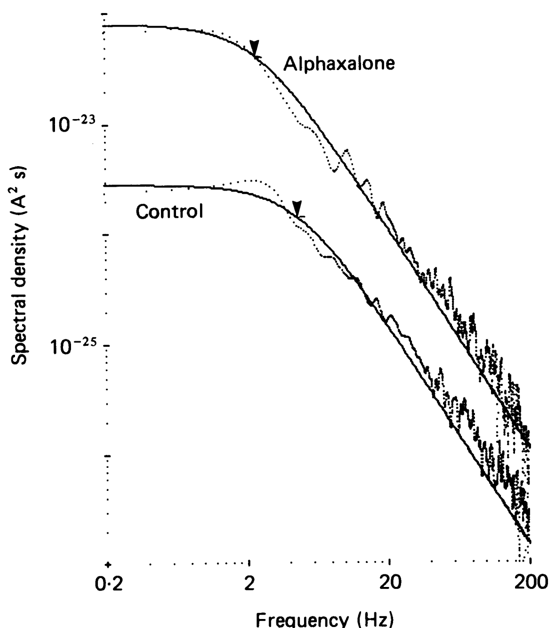

Fig. 8. Alphaxalone prolongs the mean open-time of GABA-operated Cl^- channels. Power spectra of current responses shown in Fig. 7*A* and *C*. In the presence of alphaxalone, the corner frequency of the Lorentzian fitted to the power spectrum is lower, corresponding to an increase in mean open-time of the GABA-operated Cl^- channels from 34 to 67 ms. Arrowheads indicate corner frequency, f_c.

TABLE 1. Single-channel parameters estimated from fluctuation analysis

	GABA alone		GABA+ alphaxalone		Alphaxalone	
Cell No.	τ	γ	τ	γ	τ	γ
1	33	17	90	8	—	—
2	22	15	72	17	—	—
3	34	14	67	10	—	—
4	37	15	74	12	—	—
5	26	21	76	18	133/5*	34
6	—	—	—	—	88	8
7	30	20	—	—	106	17
8	—	—	—	—	122/5*	20
Mean:	30	17	74	13	105	20

Estimates of mean open channel lifetime (τ; ms) and elementary conductance (γ; pA) obtained from spectral analysis of the current fluctuations induced by GABA or alphaxalone alone or in combination. The open-time of GABA-operated channels is clearly increased in the presence of 1 μM-alphaxalone, while the anaesthetic alone appears to open channels of long open-time relative to those activated by GABA.

* Experiments in which two Lorentzian functions were required to give an adequate fit.

Fluctuation analysis of alphaxalone-induced current responses

In four neurones, we obtained spectra for current responses to alphaxalone. In two of four recordings the spectra could not be fitted by a single Lorentzian function (e.g. Fig. 9); a good fit to the experimental data could be obtained with the sum of two

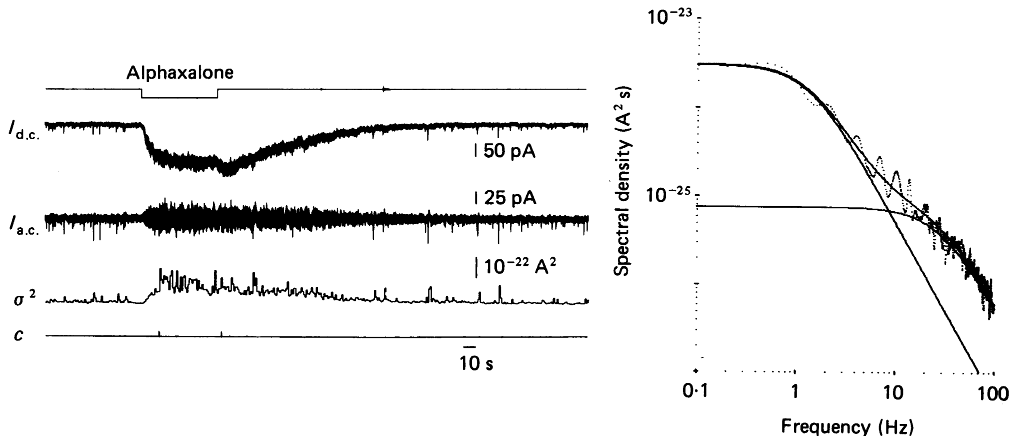

Fig. 9. Alphaxalone currents may consist of long-duration channel openings. The power spectrum for the direct alphaxalone-induced current in this neurone could not be well described by a single Lorentzian, but could be satisfactorily fitted by the sum of two Lorentzians, corresponding to channel open-times of 122 and 5 ms. The elementary conductance estimate was 20 pS, if one assumes that the majority of the power in the spectrum is contributed by the slow component (see text). The pronounced oscillations seen in this spectrum (and visible to a lesser extent in the other spectra) are also present in background spectra or spectra obtained by filtering 'white noise', and are a product of our spectral calculation procedures. For key to symbols see legend of Fig. 6.

Lorentzians, corresponding to mean open-time values of 105 and 5 ms respectively. The estimates of elementary conductance were prone to variability since this is dependent on both V_D and $S(0)$. Furthermore, the proportion of I_N contributed by the higher-frequency component is unknown and therefore γ cannot be calculated unless one assumes that this is negligible, i.e. that most of I_N is contributed by the slower component, in which case the estimates of γ obtained for current responses to alphaxalone were not significantly different from those obtained for GABA itself (Table 1).

DISCUSSION

The results described above indicate that, at low micromolar concentrations, the steroid anaesthetic alphaxalone potentiates Cl^--conductance responses to GABA in cultured rat spinal neurones, while at somewhat higher doses the drug activates Cl^- conductance in the absence of GABA. In view of the pressure application method used to apply the steroid to individual neurones, where some dilution is inevitable, it is probable that the anaesthetic modulates GABA-activated Cl^- conductance in the 0·1–1 μM range. Previous pharmacological studies in rat cuneate nucleus slices and bovine chromaffin cells have indicated that alphaxalone potentiates the actions

of GABA at bath concentrations as low as 100 nM (Harrison & Simmonds, 1984; Cottrell *et al.* 1985). The plasma concentrations of alphaxalone measured during surgical anaesthesia in man are in the range 1–10 μM (Sear & Prys-Roberts, 1979), indicating that an interaction of the drug with GABA-mediated synaptic inhibition is likely to occur at anaesthetic concentrations.

Alphaxalone selectively and stereospecifically enhances responses to GABA

The potentiation of GABA-activated Cl^- conductance by alphaxalone appears to be a relatively selective effect of the anaesthetic, since Cl^--dependent responses to another inhibitory amino-acid transmitter (glycine) are unaffected by the steroid in cultured rat spinal neurones and in rat cuneate nucleus (Harrison & Simmonds, 1984). Another pharmacological action of alphaxalone which occurs at higher concentrations (3–100 μM) is the depression of excitatory responses to acetylcholine acting on nicotinic receptors (Gillo & Lass, 1984; Cottrell *et al.* 1985).

The modulation of GABA-activated Cl^- conductance by alphaxalone is not mediated via an interaction with the benzodiazepine binding site on the GABA-receptor–Cl^--channel complex, since the benzodiazepine antagonist Ro15-1788, which blocks the potentiation of GABA-induced responses by benzodiazepines, fails to reverse potentiation of GABA-evoked responses by alphaxalone in cultured rat spinal neurones and rat cuneate nucleus (Harrison & Simmonds, 1984). Ro15-1788 also fails to reverse the enhancement of GABA responses by pentobarbitone and other anaesthetic barbiturates in cultured spinal neurones (D. G. Owen, unpublished observations).

It is well known that the 3β-hydroxy isomer of alphaxalone lacks activity as an anaesthetic, and it has been suggested that this lack of activity is related to the inability of the β-isomer to disorder neuronal membrane lipids (Lawrence & Gill, 1975). In cultured rat spinal neurones, we found that the 3β-hydroxy isomer was inactive at 10 μM in potentiating responses to GABA, consistent with results obtained in the rat cuneate nucleus slice (Harrison & Simmonds, 1984). The positive correlation between the abilities of these compounds to disorder membrane lipid and potentiate GABA might lead one to speculate that alphaxalone achieves its effects at a hydrophobic site, possibly at the interface between the GABA receptor–Cl^--channel complex and its membrane environment. However, pentobarbitone, which like alphaxalone potentiates GABA-activated Cl^- conductance at low (10–20 μM) concentrations and directly activates Cl^- conductance at high (100–200 μM) concentrations, is only active when applied to the external surface of neuronal membranes (Akaike, Hattori, Inomata & Oomura, 1985). This finding suggests that pentobarbitone acts at a site on the GABA receptor complex that is exposed at the external face of the membrane. A similar study of alphaxalone could reveal whether the steroid's action is also at an externally exposed site.

Mechanism of potentiation of GABA responses by alphaxalone

We have assumed a simple two-state (open–closed) model of the Cl^- channels activated by GABA in our study of the current fluctuations recorded under voltage clamp in the presence and absence of alphaxalone. The results of our analysis suggest that the mechanism by which alphaxalone potentiates responses to pharmaco-

logically applied GABA can be accounted for primarily in terms of an increase in the mean open-time of the Cl^- channels activated by GABA. Fluctuation analyses of potentiated responses show that the corner frequency of the calculated power spectrum is consistently shifted to a lower frequency, corresponding to an increase in the estimated duration of the microscopic channel openings underlying the macroscopic current responses recorded. An increase in the apparent open-time of channels operated by GABA is sufficient to account for the macroscopic potentiation by alphaxalone of current responses to GABA. In our experiments, a 2–3-fold increase in the amplitude of the current response to GABA was associated with a similar change in the open-time of GABA-activated Cl^- channels. An increase in GABA-activated Cl channel open-time by pentobarbitone has also been reported (Study & Barker, 1981). The increase of mean channel open-time may be related to a decrease in the dissociation rate of GABA from specific binding sites on the receptor complex. In fact, both alphaxalone (Harrison & Simmonds, 1984) and pentobarbitone (Olsen & Snowman, 1982) enhance the binding of radiolabelled GABA agonists to rat brain membranes. Pentobarbitone slows the dissociation of these radioligands from rat brain membranes (Olsen & Snowman, 1982), and it seems plausible that alphaxalone shares this property. It will be valuable to compare estimates of elementary ion-channel properties derived from fluctuation analysis with measurements of alphaxalone–GABA interactions at the single-channel level in membrane patches.

It should be noted that relatively brief (3–5 ms) openings of GABA-activated Cl^- channels can be observed in neuronal membrane patches, in addition to a population of longer openings (25–30 ms) (Jackson, Lecar, Mathers & Barker, 1982; Sakmann, Hamill & Bormann, 1983; Mathers, 1985). Fluctuation analyses of GABA-activated Cl^- currents in cultured hippocampal or cerebellar neurones using whole-cell recording techniques have also suggested the occurrence of brief channel openings (Ozawa & Yuzaki, 1984; Cull-Candy & Ogden, 1985). The amount of charge transfer attributable to these brief openings is small relative to that attributable to the longer-lasting transitions in membrane conductance. In the present experiments, the spectra of currents evoked by GABA alone and in the presence of alphaxalone were consistently well described by a single Lorentzian function, especially in the whole-cell patch recordings, where the usual band width was 0·5–500 Hz. Excess power was not observed in the high-frequency range of these spectra, in contrast to analogous recordings from cultured cerebellar neurones (Cull-Candy & Ogden, 1985). We can offer no explanation for this discrepancy at this time.

Direct membrane action of alphaxalone

A second action of alphaxalone occurred at higher steroid concentrations. In the absence of exogenously applied GABA, alphaxalone evoked a membrane potential and conductance response whose absolute polarity depended on the Cl^- gradient. This strongly suggests that the response to alphaxalone, like that evoked by GABA and (−)pentobarbitone, results from activation of Cl^- conductance. Coincident reversal of GABA- and alphaxalone-evoked membrane currents in voltage-clamped spinal neurones further supports this conclusion. A presumed Cl^--dependent response to alphaxalone has also been observed in recordings from bovine chromaffin cells (Cottrell *et al.* 1985). Our pharmacological studies of the direct alphaxalone response

showed that, like the responses to GABA and pentobarbitone (R. E. Study & J. L. Barker, unpublished observations), the response was suppressed by bicuculline and potentiated by diazepam. Fluctuation analysis of the Cl^- currents evoked by alphaxalone suggests that, like responses to pentobarbitone, they consist of apparently long-lasting channel openings of approximately 100 ms duration. In several experiments a second kinetic component of briefer (5 ms) openings was also recorded. Single-channel conductance estimates obtained from noise analysis of GABA- and alphaxalone-induced currents were not significantly different ($\sim$ 20 pS).

Since alphaxalone (like the anaesthetic barbiturates: Olsen & Snowman, 1982) does not displace, but in fact *enhances* the binding of [^{3}H]muscimol to rat brain membranes (Harrison & Simmonds, 1984), we conclude that the direct response to alphaxalone probably results from the allosteric activation of Cl^- conductance via the GABA-receptor–Cl^--channel complex. It is possible that the direct response results from potentiation of the action of GABA present in the recording medium due to the presence in the culture plate of neurones that synthesize and release GABA. Final resolution of this question would be provided by recording from cell-attached patches with alphaxalone in the recording pipette; such experiments have been undertaken with pentobarbitone, and indicate that the barbiturate is capable of opening Cl^- channels in the absence of GABA (Jackson *et al.* 1982).

Conclusions

The actions of alphaxalone at the GABA-receptor–Cl^--channel complex in rat spinal neurones are similar to those previously described for pentobarbitone in potentiating GABA (Barker & Ransom, 1978), increasing the apparent open-time of GABA-activated channels (Study & Barker, 1981), and directly activating Cl^- conductance apparently by stimulating long-duration channel openings (Mathers & Barker, 1980). The potency of alphaxalone in producing these effects is between 30 and 100 times greater than that of pentobarbitone.

Other effects of the anaesthetic barbiturates that may contribute to their C.N.S. depressant actions include a reduction of Ca^{2+}-dependent action potential duration (Heyer & Macdonald, 1982) and Ca^{2+} currents (Werz & Macdonald, 1985) and a prolongation of post-spike after-hyperpolarization (Carlen, Gurevich, Davies, Blaxter & O'Beirne, 1985). Further studies with the anaesthetic steroid should reveal whether these other membrane conductance mechanisms are also sensitive to alphaxalone, and whether there may be some common link between drug effects on such mechanisms and on the GABA-receptor–ionophore complex.

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