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Alfaxalone potentiates and mimics GABA-induced contractile responses in the guinea-pig isolated ileum

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1 Alfaxalone (1–100 nM) potentiated γ-aminobutyric acid$_A$ ($GABA_A$)-receptor-mediated contractile responses in the guinea-pig isolated ileum, with a leftward shift of the GABA concentration-response curve, and a significant potentiation of the GABA-induced contractions over the lower concentration-range for GABA (3–30 μM). Alfadalone on the other hand, did not affect contractile responses to GABA.

2 Picrotoxinin (10 μM) induced a non-parallel rightward shift of the GABA concentration-response curve, with a 50% depression of the maximum response to GABA. Alfaxalone (100 nM) potentiated the responses to GABA in the presence of picrotoxinin (10 μM) over the GABA concentration-range of 10–100 μM, causing a leftward shift of the concentration-response curve, but without affecting the depression of the maximum response by picrotoxinin.

3 Bicuculline methochloride (10 μM) caused a parallel rightward shift of the GABA concentration-response curve; the ratio of this shift was unchanged in the presence of alfaxalone (100 μM), although the latter itself displaced the curve leftwards.

4 Alfaxalone (1–100 mM) also induced a similar potentiation of contractile responses to 3-amino-1-propanesulphonic acid (3-APS), a GABA agonist not subject to uptake. Such concentrations of alfaxalone were ineffective against contractile responses to exogenous acetylcholine.

5 Higher concentrations of alfaxalone (1 μM and above), however, elicited a GABA-like ileal contraction, sensitive to both picrotoxinin (10 μM) and bicuculline (10 μM).

6 In conclusion, alfaxalone potentiated $GABA_A$-receptor-mediated contractile responses in the guinea-pig isolated ileum by acting at a modulatory site on $GABA_A$-receptor-chloride-ionophore complexes of GABA-sensitive myenteric neurones, whilst high concentrations of alfaxalone exhibited a GABA-mimetic action at $GABA_A$-receptors in the ileum. It is suggested that more than one site may exist where steroids interact with the $GABA_A$-receptor-ionophore complexes.

Introduction

Several naturally occurring steroids are either hypnotic-anaesthetics or convulsants (Selye, 1942; Heuser & Eidelberg, 1961). These actions are in general weak, requiring high doses, but systematic modification of the steroid structure has led to the development of hydroxydione (5β-pregnan-21-o1-3, 20-dione) and alfaxalone (5α-pregnan-3α-o1-11,20-dione), both of which have been used in anaesthesia (Gyermak & Soyka, 1975). Of these, the latter has received most recent attention, in particular the mechanism of its anaesthetic action. Alfaxalone prolongs γ-aminobutyric acid (GABA)-mediated inhibitory potentials in the guinea-pig olfactory cortex (Scholfield, 1980) as well as GABA-mediated dorsal root potentials associated with presynaptic inhibition in the cat (Lodge & Anis, 1984), and potentiates muscimol-induced depolarization in rat cuneate-slice preparations (Harrison & Simmonds, 1984; Simmonds *et al.*, 1984). These actions evidently involve altered chloride channel conductance at $GABA_A$-receptor-ionophore complexes (Cottrell *et al.*, 1987), with prolongation of the mean open-time of GABA-activated channels (Barker *et al.*, 1987; Harrison *et al.*, 1987a,b) in a manner resembling the effect of anaesthetic barbiturates (Schultz & Macdonald, 1981; Study & Barker, 1981). This suggests that alfaxalone modulates the GABA-induced acti-

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vation of chloride channels by acting at an allosteric site associated with $GABA_A$-receptors; in addition, higher concentrations of alfaxalone directly activate chloride channel opening in central neurones without the involvement of GABA (Harrison & Simmonds, 1984; Cottrell *et al.*, 1987; Barker *et al.*, 1987). Such modulatory interactions at $GABA_A$-receptor complexes can also be functionally examined using the isolated ileum of the guinea-pig, where barbiturates potentiate $GABA_A$-receptor-mediated contractile responses to GABA and reverse the depression of GABA-induced ileal contractions by picrotoxinin (Ong & Kerr, 1984), as well as by caprolactams acting at a similar site (Kerr *et al.*, 1986). We have therefore investigated the effect of alfaxalone on ileal GABA-induced contractions, in order to compare the actions of this steroid with those of the previously investigated barbiturates, and to gain some insight into the nature of the site(s) at which alfaxalone acts to modulate GABA-induced responses.

Methods

Guinea-pig isolated ileal preparations

Guinea-pigs of either sex, weighing between 200–400 g, were stunned by a blow on the head and bled. Segments of the distal ileum, 3–4 cm in length, were quickly removed, cleared of their intra-luminal contents and mounted vertically in a 10 ml organ bath containing modified Krebs-bicarbonate solution of the following composition (mM): Na^+ 151.0, K^+ 4.6, Mg^{2+} 0.6, Ca^{2+} 2.8, Cl^- 134.9, HCO_3^- 24.9, $H_2PO_4^-$ 1.3, SO_4^{2-} 0.6, glucose 7.7 (pH 7.4 at 37°C). The Krebs solution was continuously gassed with a mixture of 95% O_2 and 5% CO_2. Isometric contractions of the longitudinal muscle of the tissue were recorded at a resting tension of 10 mN with a Grass Model FT03 force transducer coupled to a Grass polygraph recorder.

The isolated ileal segments were allowed to equilibrate in warmed oxygenated Krebs solution for at least 60 min before any effects of drug treatments were examined on the resting tissues. The time interval between each drug application into the organ bath was 30 min, and antagonists were added at least 3 min before agonists were tested, depending on the experiment. In experiments where alfaxalone was added to test the effect on the responses induced by the agonists, alfaxalone was added at least 2 min before the agonists were applied into the bath. Control responses to GABA were always obtained before any drug application, and were re-established after washing out the drug. The volumes of drugs used were never more than 1% of the bath volume in each experiment. Student's t test for paired and unpaired samples was used to assess the significance ($P < 0.05$) of differences between mean values of the dose-response effects. EC_{50} was taken as the effective concentration of the agonist to induce a 50% response. All experiments were performed in duplicate, and were repeated at least 8 times on 8 tissues from 4 different animals.

Sources of drugs and chemicals

Acetylcholine chloride (ACh), γ-aminobutyric acid (GABA), 3-amino-1-propanesulphonic acid (3-APS), picrotoxinin (dissolved in 1 : 9 absolute alcohol and distilled water) all from Sigma; bicuculline methochloride (Pierce); alfaxalone, alfadalone (Glaxo). Both alfaxalone and alfadalone were dissolved in methanol, the final concentration of methanol in the bath being 0.001–0.01%. Such concentrations of methanol did not affect tissue baseline responses, or responses to exogenous GABA, 3-APS or ACh.

Results

Alfaxalone 100 nM reversibly potentiated ileal contractile responses to GABA (10 μM; Figure 1a). Significant potentiation ($P < 0.05$) of contractile responses to GABA (10 μM; the approximate EC_{50}) was found when using alfaxalone over the concentration range 1–100 nM (Figure 2), with a threshold between 0.1–1 nM; whilst higher concentrations of alfaxalone (1–10 μM) themselves induced a GABA-like contractile response in the ileum. Such contractile responses to higher concentrations of alfaxalone were sensitive to picrotoxinin (10 μM; Figure 1b) and to bicuculline methochloride (10 μM). Recovery of the contractile responses to GABA, back to the control level, generally occurred within 30 min after washing alfaxalone from the bath, as did recovery from the higher concentrations of alfaxalone that elicited GABA-like contractile responses. On the other hand, the related 21-acetoxy derivative, alfadalone (0.1–1000 nM) did not affect ileal contractile responses to GABA.

In the presence of alfaxalone (100 nM), there was a leftward shift of the GABA concentration-curve with a marked potentiation of the responses over the lower concentration range of GABA (3–30 μM) (Figure 3). Picrotoxinin (10 μM) induced a non-parallel rightward shift of the GABA concentration-response curve, with a 50% depression of the maximum contractile response to GABA. Alfaxalone (100 nM) still potentiated the responses to GABA in the presence of picrotoxinin (10 μM), causing a leftward shift of the depressed concentration-response curve, but did not affect ('reverse') the depression of

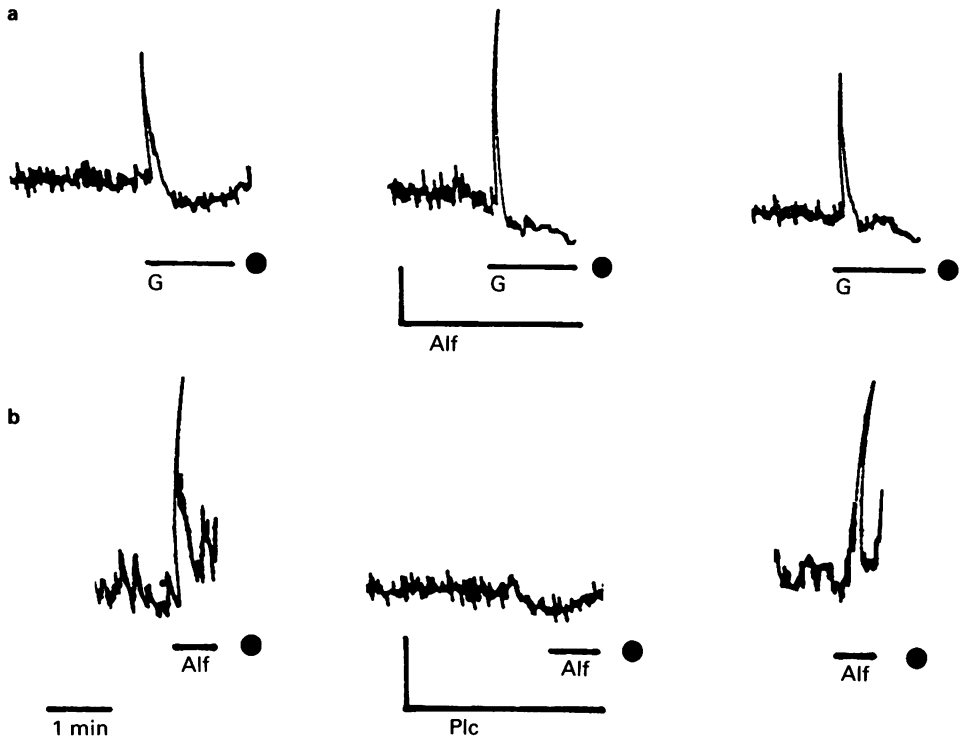

Figure 1 In the guinea-pig isolated ileum, (a) alfaxalone (Alf: 100 nM) significantly potentiated GABA (G; 10 μM)-induced contractions; recovery of the contractile response to GABA was observed after tissue wash-out (●). The number of experiments performed was $n = 12$. (b) Alfaxalone (Alf; 1 μM) itself induced a contractile response that was antagonized by picrotoxinin (Pic; 10 μM), with a recovery of the contractile response to Alf after tissue wash-out (●). The number of experiments performed was $n = 10$.

the maximum response by picrotoxininin (Figure 3). Figure 3 shows the reversal of a comparable response to GABA (300 μM) in the presence of picrotoxinin (10 μM) by a barbiturate (pentobarbitone, 0.5 mM), as previously described (Ong & Kerr, 1984). The potentiation of GABA-induced ileal contractions by alfaxalone in the presence of picrotoxinin is illustrated in Figure 4, where it can be seen that picrotoxinin (10 μM) abolished the response to GABA (10 μM), but GABA still elicited a (reduced) contraction in the combined presence of picrotoxinin and alfaxalone (100 nM); the responses to GABA alone and in the presence of alfaxalone were restored upon washout of picrotoxinin.

By contrast, bicuculline (10 μM) caused a rightward shift of the GABA concentration-response curve in a parallel manner, without affecting the maximum response to GABA. In the presence of bicuculline (10 μM), although the GABA concentration-curve was displaced to the right, the curve was again shifted leftwards in the combined presence of alfaxalone (1–10 nM) and bicuculline, to a comparable extent and without altering the slope of the curve (not shown). However, the 8 fold shift to the right for the GABA concentration-response curve, in the presence of bicuculline persisted as an 8 fold rightward shift when both bicuculline (10 μM) and alfaxalone (100 nM) were present (*vide* Ong & Kerr, 1984, Figure 3, for the comparable action of barbiturate in the presence of bicuculline). Similar potentiations by alfaxalone were also seen with 3-APS (3–30 μM), a $GABA_A$-receptor agonist with no substrate affinity for the GABA uptake system (Ong, 1987). Alfaxalone (0.01–100 nM) did not affect contractile responses to exogenous ACh (10 nM).

Discussion

Alfaxalone, over a lower concentration-range (1–100 nM), potentiated $GABA_A$-receptor-mediated contractions in guinea-pig isolated ileal preparations, whilst at higher concentrations (1 μM and above), this steroid anaesthetic itself induced a GABA-mimetic

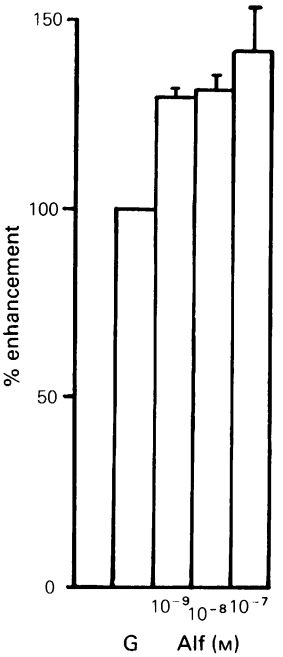

Figure 2 Potentiation of ileal contractile responses to GABA (G; 10 μM) by alfaxalone (Alf), the responses are expressed as a percentage enhancement of the control GABA response, taken as 100%. Vertical lines indicate the s.e.mean for at least 8 experiments performed for each dose of alfaxalone; the level of significance was $P < 0.05$ in each case.

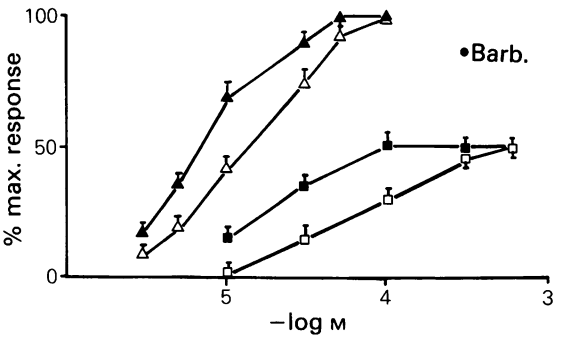

Figure 3 Concentration-response curves for GABA-induced contractile responses in the presence of picrotoxinin and alfaxalone in the guinea-pig isolated ileum. (□) Picrotoxinin (10 μM) caused a rightward shift of the concentration-response curve for (△) GABA, lowered the slope and reduced the maximum response, whilst (▲) alfaxalone (100 nM) produced a leftward shift of the concentration-response curve for GABA. In the presence of picrotoxinin (10 μM), (■) alfaxalone (100 nM) shifted the depressed GABA concentration-response curve to the left but did not alter the reduced maximum response. Picrotoxinin was left in the bath for 3–5 min, and alfaxalone for 1 min before application of GABA. (●) Barb. indicates a comparable response for GABA (300 μM) in the presence of picrotoxinin (10 μM) and pentobarbitone (0.5 mM); where pentobarbitone restored the maximum GABA-induced response toward the control, as previously found (see Ong & Kerr, 1984). Results are expressed on the ordinate scale as a percentage of the maximum contraction induced by GABA. Each point represents the mean of at least 8 experiments; vertical lines indicate s.e.mean.

contraction that was sensitive to both bicuculline and picrotoxinin. Such actions are indicative that alfaxalone acts at $GABA_A$-receptor complexes in the guinea-pig ileum. Bicuculline and picrotoxinin differed in their antagonist actions. Bicuculline brought about a comparable parallel rightward shift of the GABA concentration-response curve both in the presence and absence of alfaxalone, but did not affect the maximal response. Whereas picrotoxinin depressed the maximum and caused a non-parallel shift of the curve, an effect that was partly counteracted by alfaxalone which restored the slope of the curve toward that of the GABA control, but without altering the depression of the maximum response.

Recent studies (Barker *et al.*, 1987; Harrison *et al.*, 1987b) indicate that alfaxalone not only potentiates responses to GABA, in cultured neurones, by prolonging the duration of chloride-channel opening, but also at higher concentrations can itself induce a picrotoxinin and bicuculline-sensitive increase in chloride conductance in the absence of GABA. Judging from the sensitivity of the ileal contractions to picrotoxinin and bicuculline induced by alfaxalone, it is likely that the present results can be explained by similar mechanisms; the sensitivity to picrotoxinin indicating that the chloride-ionophores of the ileal $GABA_A$-receptor complexes are involved in these responses (c.f. Krantis & Kerr, 1981; Ong, 1987). However, it is possible that alfaxalone acts in the ileum partly by releasing GABA or inhibiting GABA-uptake, although similar potentiation by alfaxalone was seen when using 3-APS, a GABA analogue which has insignificant affinity for GABA-uptake systems (Ong, 1987). Nevertheless, the two actions of alfaxalone in the ileum, potentiation and GABA-mimetic stimulation, resemble those of barbiturates (Ong & Kerr, 1984), with the crucial difference that alfaxalone did not tend to restore the maximum response to GABA in the presence of picrotoxinin, a non-competitive inhibitor of GABA (Krantis & Kerr, 1981), as happened with barbiturates. Harrison & Simmonds (1983) have already observed this effect with alfaxalone in the cuneate slice. Also they have drawn attention to the dissociation of potentiation of responses to GABA by

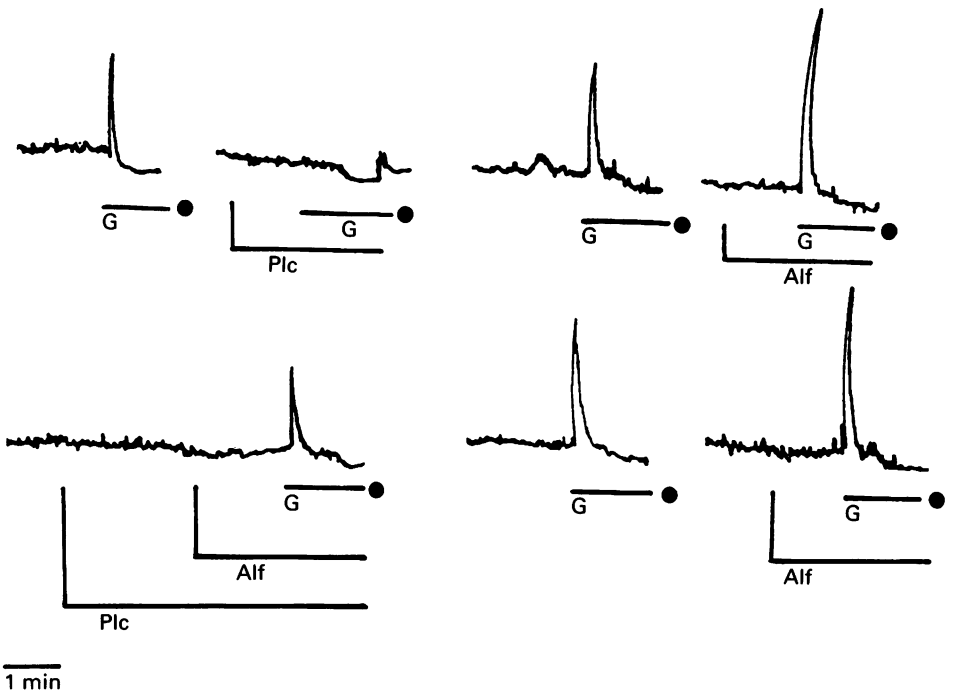

Figure 4 Effects of picrotoxinin (Pic) and alfaxalone (Alf) on GABA-induced contractile responses in the guinea-pig isolated ileum. Picrotoxinin (10 μM) completely antagonized the contraction induced by GABA (G; 10 μM) without affecting the 'after-relaxation'. Alfaxalone (100 nM) potentiated the GABA-induced contraction, and partially restored the GABA-induced contractile response in the presence of picrotoxinin (10 μM). Upon tissue wash-out indicated by (●), the GABA-induced contractile response remained potentiated by alfaxalone (100 nM). The number of experiments performed was $n = 8$.

anaesthetic barbiturates, in the presence and absence of picrotoxinin, and the ability of agents such as chlormethiazole and anticonvulsant barbiturates to reverse the picrotoxinin-induced depression of maximal responses to GABA whilst showing minimal potentiating action (Harrison & Simmonds, 1983). In this regard, alfaxalone resembles anaesthetic barbiturates rather than the latter anticonvulsants.

Although the anaesthetic actions of alfaxalone may be substantially dependent on the GABA-mimetic properties of alfaxalone which appear at higher concentrations (>1 μM), close to those found in the brain during anaesthesia (Sear & Prys-Roberts, 1979; Barker *et al.*, 1987), this steroid also modulates GABA-induced actions when used at lower concentrations (Harrison & Simmonds, 1984). The threshold for this potentiating effect in chromaffin cells was close to 30 nM (Cottrell *et al.*, 1987), whereas substantial potentiation of GABA-induced ileal contractions was already found here at 1 nM, the threshold lying between 0.1 and 1 nM. In other preparations, such potentiation of GABA-induced responses is in some way related to modulation by alfaxalone of $GABA_A$-receptor-ionophore- complexes; alfaxalone increases the affinity of [^{3}H]-muscimol at low affinity binding sites (Harrison & Simmonds, 1984), and interacts with GABA in displacing [^{35}S]-t-butylcyclophosphorothionate from the convulsant site at the ionophore-complexes in a bicuculline-sensitive manner (Gee *et al.*, 1987). Similar steroid interactions have also been observed at the barbiturate site of $GABA_A$-receptor complexes (Majewska *et al.*, 1986). Whilst aspects of these interactions resemble those of barbiturates, as do the actions of alfaxalone on chloride conductances in isolated neurones (Harrison *et al.*, 1987b), the direct actions of alfaxalone in activating chloride channel conductance can themselves be potentiated by benzodiazepines and barbiturates (Barker *et al.*, 1987; Cottrell *et al.*, 1987), suggesting that alfaxalone modulates GABA-induced responses at some site other than that affected by barbiturates. The present results confirm that alfaxalone exerts two effects at $GABA_A$-receptor-ionophore complexes, low concentrations potentiating GABA-induced responses,

whilst high concentrations elicit direct GABA-mimetic responses. This latter action is unique in our experience; using a considerable range of steroids it has been found that increasing concentrations of many steroids depress ileal contractile responses to GABA in the manner of a non-competitive antagonist, rather than exhibit GABA-mimetic actions, e.g. cortisol is 1000 times as potent as alfaxalone in potentiating ileal responses to GABA but acts as an antagonist at 10 nM and above (Ong *et al.*, 1987). The potentiating and mimetic actions of alfaxalone, then, probably arise from effects at different sites; the potentiating effect being shared by a number of steroids whereas the mimetic action is associated with $GABA_A$-receptors since, like that of barbiturates, it is sensitive to bicuculline which is a competitive antagonist acting at these recognition sites in the ileum (Ong & Kerr, 1984). Such findings, taken together with the present results, suggest that steroids in general may act at more than one site capable of modulating GABA-induced alterations in chloride conductance of GABA-sensitive neurones.

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References

BARKER, J.L., HARRISON, N.L., LANGE, G.D. & OWEN, D.G. (1987). Potentiation of gamma-aminobutyric acid-activated chloride conductance by a steroid anaesthetic in cultured rat spinal neurones. *J. Physiol.*, **386**, 485–501.

COTTRELL, G.A., LAMBERT, J.J. & PETERS, J.A. (1987). Modulation of $GABA_A$ receptor activity by alphaxalone. *Br. J. Pharmacol.*, **90**, 491–500.

GEE, K.W., CHANG, W-C., BRINTON, R.E. & McEWEN, B.S. (1987). GABA-dependent modulation of the chloride ionophore by steroids in rat brain. *Eur. J. Pharmacol.*, **136**, 419–423.

GYERMEK, L. & SOYKA, L.F. (1975). Steroid anaesthetics. *Anesthesiology*, **42**, 331–344.

HARRISON, N.L. & SIMMONDS, M.A. (1983). Two distinct interactions of barbiturate and chlormethiazole with the $GABA_A$-receptor complex in rat cuneate nucleus *in vitro*. *Br. J. Pharmacol.*, **80**, 387–394.

HARRISON, N.L. & SIMMONDS, M.A. (1984). Modulation of the GABA receptor complex by a steroid anaesthetic. *Brain Res.*, **323**, 287–292.

HARRISON, N.L., MAJEWSKA, M.D., HARRINGTON, J.W. & BARKER, J.L. (1987a). Structure-activity relationships for steroid interaction with the gamma-aminobutryic $acid_A$ receptor complex. *J. Pharmacol. Exp. Ther.*, **241**, 346–353.

HARRISON, N.L., VICINI, S. & BARKER, J.L. (1987b). A steroid anesthetic prolongs inhibitory post-synaptic currents in cultured rat hippocampal neurons. *J. Neurosci.*, **7**, 604–609.

HEUSER, G. & EIDELBERG, E. (1961). Steroid-induced convulsions in experimental animals. *Endocrinology*, **69**, 915–924.

KERR, D.I.B., ONG, J., PRAGER, R.H. & WARD, D.A. (1986). Caprolactam-barbiturate interaction at the $GABA_A$ receptor complex in the guinea-pig intestine. *Eur. J. Pharmacol.*, **124**, 203–206.

KRANTIS, A. & KERR, D.I.B. (1981). GABA induced excitatory responses in the guinea-pig small intestine are antagonised by bicuculline, picrotoxin and chloride ion tured central neurons. *Proc. Natl. Acad. Sci., U.S.A.* **78**, 7180–7184.

LODGE, D. & ANIS, N.A. (1984). Effects of ketamine and three other anaesthetics on spinal reflexes and inhibitions in the cat. *Br. J. Anaesth.*, **56**, 1143–1151.

MAJEWSKA, M.D., HARRISON, N.L., SCHWARTZ, R.D., BARKER, J.L. & PAUL, S.M. (1986). Steroid hormone metabolites are barbiturate-like modulators of the GABA receptor. *Science*, **232**, 1004–1007.

ONG, J. (1987). Uptake inhibitors potentiate gamma-aminobutyric acid-induced contractile responses in the isolated ileum of the guinea-pig. *Br. J. Pharmacol.*, **91**, 9–15.

ONG, J. & KERR, D.I.B. (1984). Potentiation of $GABA_A$-receptor-mediated responses by barbiturates in the guinea-pig ileum. *Eur. J. Pharmacol.*, **103**, 327–332.

ONG, J., KERR, D.I.B. & JOHNSTON, G.A.R. (1987). Cortisol: a potent biphasic modulator at $GABA_A$-receptor-complexes in the guinea-pig isolated ileum. *Neurosci. Lett.*, **82**, 101–106.

SCHOLFIELD, C.N. (1980). Potentiation of inhibition by general anaesthetics in neurones of the olfactory cortex *in vitro*. *Pflugers Arch.*, **383**, 249–255.

SCHULZ, D.W. & MacDONALD, R.L. (1981). Barbiturate enhancement of GABA-mediated inhibition and activation of chloride ion conductance: correlation with anticonvulsant and anaesthetic actions. *Brain Res.*, **209**, 177–188.

SEAR, J.W. & PRYS-ROBERTS, C. (1979). Plasma concentrations of alphaxalone during continuous infusion of althesin. *Br. J. Anaesth.*, **51**, 861–865.

SELYE, H. (1942). Correlations between the chemical structure and the pharmacological actions of the steroids. *Endocrinology*, **30**, 437–453.

SIMMONDS, M.A., TURNER, J.P. & HARRISON, N.L. (1984). Interactions of steroids with the $GABA_A$-receptor complex. *Neuropharmacology*, **23**, 877–878.

STUDY, R.E. & BARKER, J.L. (1981). Diazepam and (−) pentobarbital-fluctuation analysis reveals different mechanisms for potentiation of GABA responses in cul-blockers. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **137**, 257–261.

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