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Anaesthetic suppression of transmitter actions in neocortex

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1 The effects of general anaesthetics were investigated on neuronal sensitivities to transmitter substances, which were determined by iontophoretic applications of acetylcholine, glutamate, N-methyl-D-aspartate (NMDA) and γ-aminobutyrate (GABA) during intracellular recording in *in vitro* slice preparations of neocortex (guinea-pig).

2 In most of the 65 neurones studied, perfusion of isoflurane (0.5–2.5 minimum alveolar concentration (MAC)) or Althesin (25–200 μM) and, in some cases, halothane (0.5–2 MAC), markedly reduced the depolarizing responses and associated membrane conductance changes evoked by dendritic applications of acetylcholine, glutamate, NMDA and GABA.

3 The order of depression was acetylcholine > glutamate or NMDA ≫ GABA. This selectivity could also be assessed from the EC_{50} for the isoflurane-induced depression of the just-maximal responses to acetylcholine, which was 0.9 MAC compared with an EC_{50} = 1.9 MAC for the suppression of glutamate responses. The selectivity was less pronounced in the case of the actions of Althesin, where the EC_{50}s were 75 μM for the depression of acetylcholine responses and 90 μM for the depression of glutamate responses.

4 The hyperpolarizing responses observed when GABA was applied near the perikaryon in 7 neurones, were slightly reduced (∼15%) in 4, and unchanged in 3 neurones during anaesthetic application.

5 The pronounced depression of the responsiveness to the putative arousal transmitters and an observed blockade of acetylcholine-induced potentiation of glutamate actions suggest that anaesthetics produce unconsciousness, at least in part, by interfering with subsynaptic mechanisms of neocortical activation.

Introduction

The unconcious state produced by anaesthetic agents can be attributed to depressant actions at excitatory synapses and to a potentiation of inhibitory postsynaptic potentials (i.p.s.ps), particularly those mediated by γ-aminobutyrate (GABA; Richards, 1983). A depression of excitatory postsynaptic potentials (e.p.s.ps), has been observed in neocortical neurones on administration of either isoflurane or Althesin (El-Beheiry & Puil, 1989b). The depression of e.p.s.ps may be a consequence of reductions in presynaptic synthesis (Cheng & Brunner, 1978) and release of transmitter (Zorychta & Čapek, 1978; Quastel & Saint, 1986) or, may be attributed to alterations in postsynaptic transmitter actions (Catchlove *et al.*, 1972; Richards & Smaje, 1976; Barker & Ransom, 1978; Lambert & Flatman, 1981; Sawada & Yamamoto, 1985), as in the case of the enhanced i.p.s.ps (Scholfield, 1980; Gage & Robertson, 1985; Barker *et al.*, 1987).

The effects of anaesthetics on the electrical excitability of the neuronal membrane are often small (Nicoll & Madison, 1982; Berg-Johnsen & Langmoen, 1987; Puil & Gimbarzevsky, 1987; El-Beheiry & Puil, 1989a) or are not always observed when the e.p.s.ps are diminished (Takahashi & Takenoshita, 1987; Miu & Puil, 1989). These lines of evidence suggest that isoflurane or Althesin may interfere with the responsiveness of the subsynaptic membrane to transmitters likely to be involved in behavioural arousal (Krnjević, 1974; Krnjević & Puil, 1975; El-Beheiry & Puil, 1989b). This possibility was assessed in the present investigations from the effects of anaesthetics on neocortical neurones which are likely sites in the production of the unconscious state (Krnjević, 1974; Savaki *et al.*, 1983). Possible alterations in the actions of the transmitters – acetylcholine, glutamate and GABA — were investigated because of their long suspected roles in neocortical activation (Jasper *et al.*, 1965; Celesia & Jasper, 1966; Shute & Lewis, 1967; Krnjević, 1974; Lysakowski *et al.*, 1989). Some of these results have been published in a preliminary form (El-Beheiry & Puil, 1989c).

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Methods

Slice preparations and recording

Sensorimotor and anterior cingulate cortices were identified in the halothane-anaesthetized guinea-pig, excised, and cut into 500 μm thick slices according to previously described procedures (El-Beheiry & Puil, 1989a). The slices were submerged in artificial cerebrospinal fluid (CSF) oxygenated with a 95% O_2/5% CO_2 mixture and kept at room temperature until required for recording (bath temperature, 32–34°C). The constituents of the artificial CSF were (in mM): NaCl 124, KCl 3.75, KH_2PO_4 1.25, $MgSO_4 . 7H_2O$ 2, $CaCl_2 . 2H_2O$ 2, dextrose 10 and $NaHCO_3$ 26. The techniques for conventional intracellular recording using 3 M KCl or 0.6 M K_2SO_4 microelectrodes and measurement of input resistance in neurones of neocortical slices in a submersion type of chamber have also been described (El-Beheiry & Puil, 1989a).

Experimental procedures

The effects of anaesthetics were assessed by the following procedures: (1) after stable recording conditions were achieved, the iontophoretic current was adjusted in the range 30–350 nA (typically 80 nA), such that a submaximal (∼ED_{50}) or a just-maximal response could be elicited with a transmitter substance; (2) the responses of relatively constant amplitude (usually 10–20 mV) were evoked at appropriate intervals (10–30 s) for a period of at least 5–8 min; (3) equi-amplitude responses to 2 or more transmitter substances were evoked in the same neurone; (4) perfusion of the anaesthetic was commenced during the continuing, intermittent transmitter applications; and (5) 3 or more responses to each transmitter substance obtained under each condition were averaged for the quantitative comparisons.

A slightly different procedure was used for investigation of NMDA responses because of the rapid development of desensitization or tachyphylaxis in the large pyramidal (layer IV–V) neurones. If a neurone was responsive to a low dose of NMDA the just-maximal effect was determined and 3 or 4 equi-amplitude responses were obtained at 3 min intervals before anaesthetic application was commenced.

Drugs and their applications

Isoflurane (Anaquest, Pointe Claire, Quebec) and halothane (Ayerst Laboratories, Montreal, Quebec) were vaporized at a $1 \, l \, min^{-1}$ flow rate with 95% O_2/5% CO_2 gas mixture by 5 Fluotec Mark 3 vaporizers (Cyprane Limited, Keighley, Yorkshire) and bubbled into 5 reservoirs containing artificial CSF for at least 25 min. These agents were then perfused at the designated minimum alveolar concentration (MAC) values (cf. Miu & Puil, 1989). Althesin (Glaxo Canada Limited), a twin steroid preparation, was administered by perfusion. Althesin contained alphaxalone and alphadalone (one-half the anaesthetic potency of alphaxalone) dissolved in cremophor EL in a 3:1 ratio.

Acetylcholine (0.5 M, pH 4.5), glutamate (0.5 M, pH 8.5), N-methyl-D-aspartate (NMDA; 100 mM, pH 9) and GABA (100 mM, pH 4.5) (all obtained from Sigma Chemical Co., U.S.A.), or NaCl for control applications, were administered by iontophoresis from a 3- or 5-barrelled micropipette which was either glued in parallel to the recording electrode at an intertip distance of $\sim 30 \, \mu m$ for somatic applications or, inserted separately into the dendritic region at a distance of $\sim 50 \, \mu m$ from the presumed location of neuronal impalement. Except for NMDA, these substances were applied at recurring intervals (10–60 s) before, during and after concomitant perfusion of an anaesthetic. The duration of application was usually 4 or 5 s for NMDA or GABA, 6 s for glutamate and 7 to 20 s for acetylcholine. In $\sim 75\%$ of the neurones, tetrodotoxin (TTX; 1–1.5 μM; Sigma Chemical Co.) was applied to block spikes and hence indirect actions of the transmitter substances on neighbouring neurones.

Statistical analysis

Results are presented as mean values $\pm$ s.e.mean. Statistical analysis was performed by Student's t test. Overall significance was determined by ANOVA.

Results

The effects of isoflurane or Althesin were investigated in 65 neurones that exhibited stable resting potentials for periods of 0.5–5 h. The interactions of halothane were examined in 10 of the neurones. The mean resting potential (V_m) and input resistance (R_i) were -69 ± 2.3 mV and $51 \pm 2.5 \, M\Omega$, respectively ($n = 65$). All neurones fired short duration spikes (>75 mV amplitude) in response to depolarizing current pulse injections. Spontaneous discharge was observed in 23% of the 65 neurones.

As previously demonstrated (El-Beheiry & Puil, 1989a; Miu & Puil, 1989), the anaesthetics produced no significant alterations in V_m, R_i or membrane time constant except when applied at the highest doses. Application of isoflurane (2.5 MAC), halothane (2.5 MAC), or Althesin (200 μM) but not the vehicle, cremophor EL (cf. Pennefather *et al.*, 1980), hyperpolarized neurones by 3–5 mV and decreased R_i by 15–20%. In such cases, d.c.-injection was employed to maintain a constant V_m for observations of interactions with transmitters.

Acetylcholine responses

Acetylcholine applied to the apical dendritic region evoked a slowly developing depolarization (12.3 ± 1.5 mV) in 36 out of 50 neurones (Figure 1). The responses were associated with increased R_i (e.g., $\sim 30\%$ in Figures 1 and 3a) and were maximal in voltage for 10–30 s after termination of the application. In 3 neurones, the responses were blocked by the application of scopolamine (20 μM), suggesting actions of acetylcholine at muscarinic receptors (cf. Krnjević, 1987). An additional feature was the appearance of postsynaptic potentials (mostly e.p.s.ps) on the falling phase of the depolarizations, even during concomitant application of TTX. This suggested an involvement of Ca-spikes or presynaptic actions at nicotinic receptors on nerve terminals (Rovira *et al.*, 1983). In 14 neurones, the acetylcholine-evoked depolarization was preceded by a transient hyperpolarization (Figure 1a) that could be blocked by application of TTX.

Anaesthetic administration reversibly depressed the depolarizing and conductance responses to acetylcholine in almost all neurones (Table 1). These effects were observed even after low doses (0.5–1 MAC) of isoflurane. Recovery was usually observed 8–10 min after such applications (Figures 1 and 3a). At least partial recovery was evident after application of a high dose of isoflurane (2–2.5 MAC) and Althesin (150–200 μM).

Glutamate and NMDA responses

All 65 neurones were depolarized by 20 ± 1.4 mV on application of glutamate. The responses were reduced by administration of isoflurane (0.5–2.5 MAC), halothane (1.5 MAC) or Althesin (25–200 μM) and typical effects of isoflurane and Althesin are illustrated in Figure 2, and summarized in Table 1. Recovery from an application of isoflurane was observed after 6–18 min and was usually more rapid than after Althesin

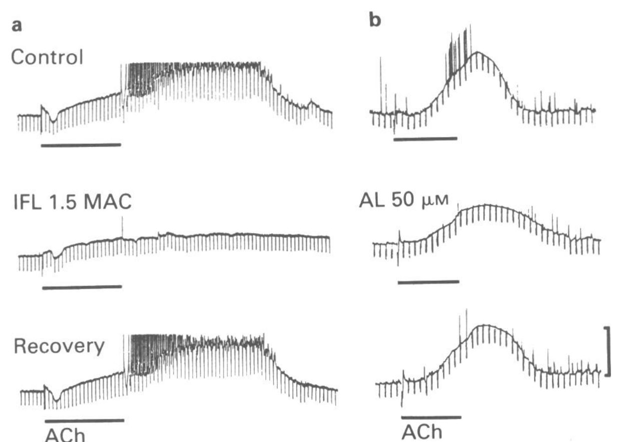

Figure 1 Depression by isoflurane (IFL) and Althesin (AL) of responses to acetylcholine (ACh; 100 nA for 20 s in (a), 125 nA for 9 s in (b) in 2 neurones (a), $V_m = -68$ mV; (b), $V_m = -72$ mV. Middle records were obtained at 9 min in (a) and 6 min in (b) of anaesthetic application. Negative voltage deflections represents tests for changes in input resistance. MAC = minimum alveolar concentration.

Table 1 Anaesthetic-induced depression of neuronal responses to transmitter substances

	Depolarization evoked by			GABA evoked	
Anaesthetic applied	*ACh* ($n = 36$)	*Glu* ($n = 65$)	*NMDA* ($n = 15$)	*hyperpolarization* ($n = 7$)	*depolarization* ($n = 29$)
Isoflurane	18/18	30/40	8/10	4/7	10/17
Althesin	15/16	25/32	7/9		11/15
Halothane	2/2	5/7			2/4

Depression was defined as >15% attenuation of control (just-maximal) response by the anaesthetic. The total number of neurones investigated was 65, i.e., all neurones were exposed to glutamate (Glu) and to at least one other transmitter substance. The anaesthetic doses were 0.5–2.5 minimum alveolar concentration (MAC) for isoflurane, 25–200 μM for Althesin and 0.5–2 MAC for halothane. ACh = acetylcholine and NMDA = N-methyl-D-aspartate.

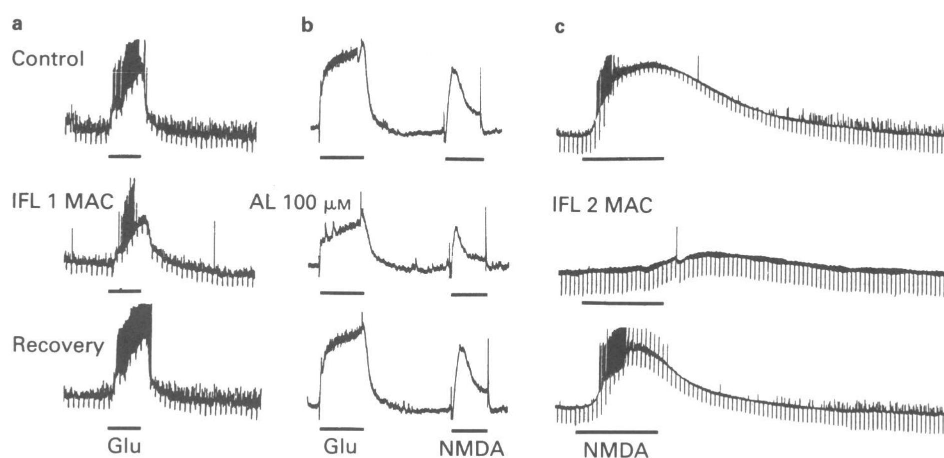

Figure 2 Anaesthetic depression of responses to glutamate (Glu; 100 nA for 5 s in (a) and 35 nA in (b)) and N-methyl-D-aspartate (NMDA; 55 nA for 5 s in (b), 110 nA for 15 s in (c)) in 2 neurones (V_m = −70 mV in (a), −75 mV in (b), −69 mV in (c)). Middle traces were obtained at ~10 min of anaesthetic application. Resistance test pulses were applied in (a) and (c). Tetrodotoxin (1.5 μM) was applied in (b). Voltage calibration: 25 mV in (a), 20 mV in (b), 30 mV in (c). For key to abbreviations used see legend of Figure 1.

application. Recovery from the depression due to the application of Althesin was observed after 15 min.

Only 15 of 37 neurones were depolarized by the application of NMDA (Figure 2b,c). In 4 of these, the responses to NMDA did not fade, even during relatively long applications (cf. Figure 2c). All NMDA responses were reduced by application of isoflurane (1–2 MAC) or Althesin (75–200 μM). These effects (Table 1) had magnitudes and recovery times similar to those for the depression of glutamate responses.

GABA responses

GABA application near the perikaryon evoked hyperpolarizing responses of 5–10 mV in 7 out of 7 neurones. Isoflurane application at 1 MAC had no significant effects on such responses, although at 2 MAC a slight reduction (~15%) was observed in 4 neurones.

When GABA was applied at a dendritic location just-maximal depolarizing responses (22.5 ± 2.0 mV) were observed in 29 out of 29 neurones (Figure 3a). The peak depolarizations which were accompanied by a decreased R_i (~50%) either waned, or maintained a constant amplitude during the application. Relatively low dose applications of isoflurane (0.5–1 MAC), or Althesin (25–100 μM) did not greatly alter these responses (Figure 3a). The maximal depression was ~40% with 2.5 MAC isoflurane, 1.5 MAC halothane or 200 μM Althesin (cf. Table 1).

Selectivity in depression

Anaesthetic interactions with transmitters When acetylcholine, glutamate and/or GABA were applied alternately to the same neurone, application of isoflurane, halothane or Althesin markedly attenuated the responses to acetylcholine after ~8 min, slightly reduced those to glutamate and produced only small changes in the depolarizing effects of GABA (cf. Figure 3 for isoflurane and Althesin). The selectivity was much more pronounced with isoflurane than Althesin application and no such effects were observed with applications of cremophor EL (Pennefather *et al.*, 1980; Cullen & Martin, 1982). In 7 neurones, low dose administration of isoflurane or Althesin produced no depression of the glutamate depolarization and sometimes prolonged its duration (cf. Figure 3b). In 5 neurones, the reductions in the acetylcholine responses were evident at 30–90 s i.e., before attenuation of glutamate responses. Full recovery from the reduction in glutamate depolarization was usually observed several minutes earlier than a complete return of the acetylcholine responses to control amplitude.

Dose-response relationships The dose-response relationships for the actions of isoflurane and Althesin on the just-maximal responses evoked by the transmitter substances are shown in Figure 4. The EC_{50} for the isoflurane depression of the acetylcholine responses was 0.9 MAC compared with 1.9 MAC for

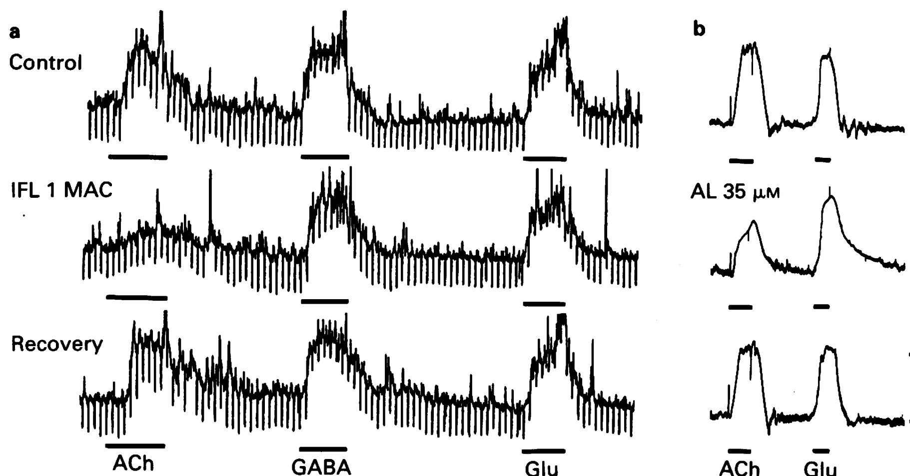

Figure 3 Effects of anaesthetic application on equi-amplitude responses to γ-aminobutyric acid (GABA; 75 nA for 6 s in (a)), glutamate (Glu; 85 nA in (a), 70 nA in (b)) and acetylcholine (ACh, 90 nA for 10 s in (a), 110 nA for 7 s in (b)) in 2 neurones (V_m = −75 mV in (a), −70 mV in (b)). Middle traces in (a) and (b) were obtained at 9 min of anaesthetic application. Resistance test pulses were applied in (a). Tetrodotoxin (1 μM) was applied in (b). Voltage calibration: 20 mV in (a), 10 mV in (b). For key to abbreviations used see legend of Figure 1.

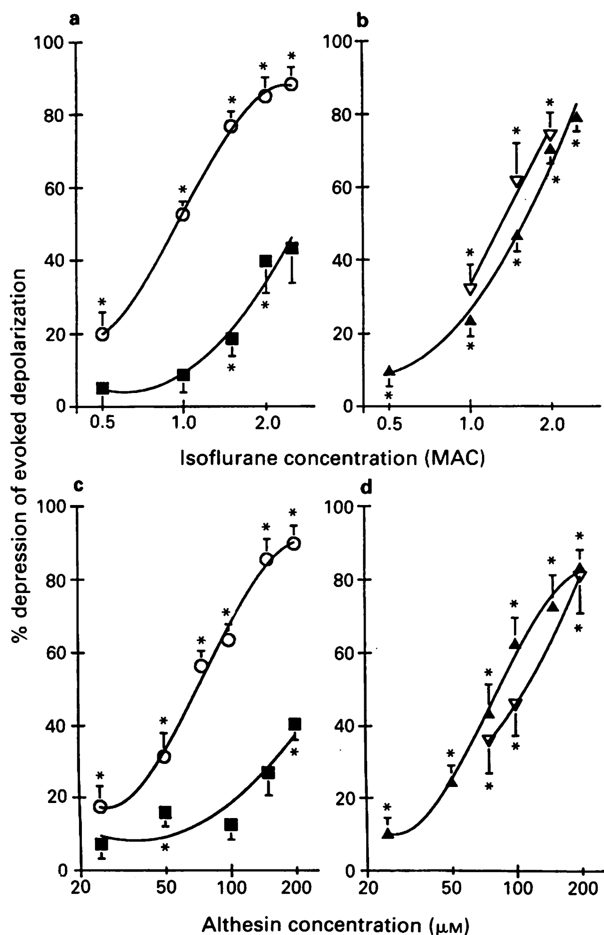

Figure 4 Pooled data from 42 neurones show dose-response relationships for (a,b) isoflurane- and (c,d) Althesin-induced depressions of depolarizations evoked by transmitter substances. Each point on the curve is the mean response from a neurone to at least 4 applications of isoflurane ($n = 22$) or Althesin ($n = 20$). * Indicates significant difference from control at $P < 0.05$. Overall significance was determined by ANOVA. Vertical lines show s.e.mean. In (a) and (c): (○) acetylcholine, (■) γ-aminobutyric acid. In (b) and (d): (▽) N-methyl-D-aspartate, (▲) glutamate.

the suppression of glutamate responses. Such differences in selectivity were also apparent in comparison with NMDA responses, although the data are more limited. The selectivity was less pronounced in the case of Althesin where the EC_{50} values were 75 μM for depression of the acetylcholine responses and 90 μM for depression of glutamate responses (Figure 4). The maximal reductions of the depolarizing responses induced by application of GABA were only about 40% at 2.5 MAC isoflurane or 200 μM Althesin.

Possible anaesthetic depression of the potentiation of glutamate-responses by acetylcholine This was examined in 7 neurones by briefly applying glutamate pulses ($\sim ED_{50}$) during the application of acetylcholine ($\sim ED_{50}$), and then concomitantly administering the anaesthetic for 6–9 min. As can be seen from Figure 5, the application of acetylcholine gradually depolarized the neurones and then enhanced and prolonged the effects of glutamate; when 1.5 MAC isoflurane ($n = 4$) or 75 μM Althesin ($n = 3$) was additionally administered, the effects of acetylcholine, including the enhancement were no longer evident. Note that such applications of anaesthetic also reduced the glutamate responses which recovered with the effects of acetylcholine.

Discussion

The unusual muscarinic actions of acetylcholine and its interactions with glutamate and anaesthetics led Krnjević (1974; 1987) to propose a depression of cholinergic activation in neocortex by the reticular activating system and intracortical transmission, as a basis for the anaesthetic state. Although the initial extracellular observations on the effects of systemic administration of various anaesthetics on cortical neurones in *in vivo* feline preparations were suggestive of this hypothesis (Catchlove *et al.*, 1972; Krnjević & Puil, 1975), there has not previously been sufficient evidence *a priori* for this mechanism. Similar *in vitro* studies on olfactory cortex showed that halothane, administered in the gas stream over the upper surface of the slice, or alphaxalone (the main active steroid in Althesin), applied in the saline perfusion as a liposome suspension, reduced glutamate-, and not acetylcholine-evoked neuronal firing (Richards & Smaje, 1976; Smaje, 1976). The reasons for this disparity probably relate to the differences in the

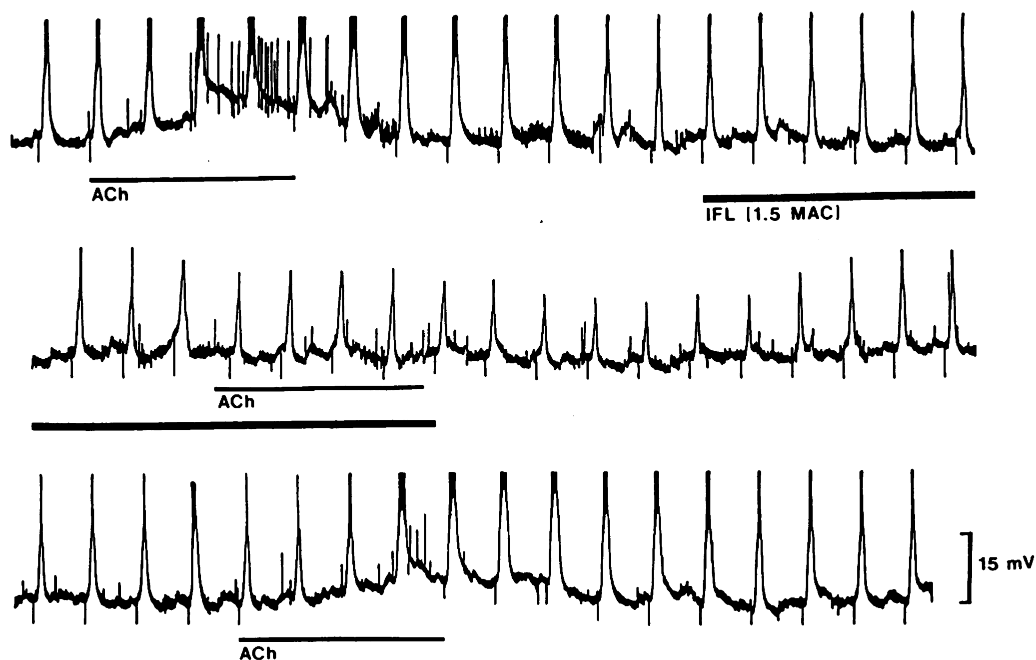

Figure 5 Continuous record of isoflurane (IFL)-induced depression of acetylcholine (ACh)-potentiation of glutamate (Glu)-induced actions on a sensorimotor neurone. Depolarizations evoked by Glu (cf. negative artefacts at onset of iontophoretic 70 nA current) and ACh (50 nA) are ~50% of their respective maximal responses. IFL was applied for 6 min (thick bar).

modes of anaesthetic application. Some recent investigations of neurones of the lateral geniculate nucleus have demonstrated that the enhancement of spontaneous spike discharge produced by electrical stimulation of the mesencephalic reticular formation, or acetylcholine application, is much more sensitive than that produced by glutamate- or NMDA-application, to the depressant effects of anaesthesia with N_2O and pentobarbitone or with N_2O and halothane (Francesconi *et al.*, 1988).

The present results in neocortical slices *in vitro* also show a degree of selectivity in the depression of arousal transmitter actions after administration of isoflurane, halothane and Althesin, i.e., the order of depression was acetylcholine > glutamate (or NMDA) $\gg$ GABA. Because changes in passive membrane properties of the neurones were not usually induced by anaesthetic application, the suppression may result from postsynaptic actions on the ionic channel-receptor complexes or on internal modulation of a secondary messenger system, subsequent to agonist-receptor interactions.

Acetylcholine responses

There are several ways in which anaesthetics can selectively interfere with the muscarinic system in cortical neurones. Anaesthetic application decreases the affinity of the receptor G-protein complexes for guanine nucleotides (Dennison *et al.*, 1987) and modifies channel proteins, thereby decreasing the number of activatable channels (Arimura & Ikemoto, 1986; Ikemoto *et al.*, 1988). Another explanation is that anaesthetics may bind allosterically with the receptor for acetylcholine and inhibit its specific binding (Young & Sigman, 1981).

Glutamate responses

The depression of the glutamate- or NMDA-evoked depolarizations by anaesthetics (Barker & Ransom, 1978; Lambert & Flatman, 1981; Sawada & Yamamoto, 1985; Thomson *et al.*, 1985; MacDonald *et al.*, 1987) may also be attributed to blocked (MacDonald *et al.*, 1987) or desensitized states of receptor-channel complexes (see Ikemoto *et al.*, 1988). These depolarizations result, at least in part, from a transmembrane Ca^{2+}-influx (Murphy & Miller, 1988; Puil & Benjamin, 1988). Anaesthetics may reduce the depolarization either directly by preventing receptor-activated Ca^{2+}-entry or, indirectly, by uncoupling Ca^{2+}-entry from its dependency on membrane voltage (Krnjević & Puil, 1988; Puil & Baimbridge, 1989).

GABA responses

In the neocortex, GABA application to perikarya elicited hyperpolarizing responses that were unaffected by isoflurane, whereas GABA application to dendrites resulted in depolarizing responses that were depressed by high concentrations of isoflurane or Althesin. GABA-evoked depolarizations have been demonstrated in pyramidal and granule neurones of hippocampal slice preparations; the ionic mechanism for the depolarization is unknown, but may involve an increased Cl-conductance as in the generation of the hyperpolarization (Alger & Nicoll, 1982; Blaxter & Carlen, 1988). Isoflurane or Althesin administration to neocortical neurones suppressed the depolarizations evoked by GABA, as observed in other neurones during application of alphaxalone as well as high doses of other anaesthetics (Cullen & Martin, 1982; Brooks *et al.*, 1986).

A potentiation of Cl^--dependent responses to GABA has been observed in olfactory cortical (Scholfield, 1980) and spinal (Barker *et al.*, 1987) neurones on application of alphaxalone in low doses (1–10 μM); these doses correspond approximately to plasma concentrations in anaesthetized patients (Sear & Prys-Roberts, 1979). Preliminary investigations have revealed a brain concentration of 60 μM alphaxalone after systemic administration of Althesin to experimental animals (Smith *et al.*, 1974). In the absence of confirmatory data, the relevance of the concentrations of alphaxalone used in the above and the present studies to the clinical state remains unknown. However, it may be significant that the cremophor EL vehicle which did not have noticeable effects on neocortical neurones in our experiments, has been shown to reduce greatly the synaptosomal membrane:buffer partition coefficient of highly lipid soluble drugs (Roth & Williams, 1979); such observations may account for the higher EC_{50}s of alphaxalone found in the present (cf. Figure 4) and previous investigations (Pennefather *et al.*, 1980).

Significance

Given the special involvement of muscarinic pathways in determining conscious processes neocortical activation would be particularly susceptible to the depressant actions of anaesthetics (Shute & Lewis 1967; Krnjević, 1974; 1987). A sharp depression of cholinergic activity in the cortex could produce a 'surgical level' of anaesthesia, whereas deeper levels could reflect a more generalized impairment in cholinergic modulation of glutamatergic transmission. The anaesthetic depression in subsynaptic responsiveness to these putative arousal transmitters would arrest neocortical expression of the conscious state.

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