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Inhibition by halothane of potassium-stimulated acetylcholine release from rat cortical slices

R. Griffiths, [1]J.M.C. Greiff, J. Haycock, C.D. Elton, D.J. Rowbotham & *R.I. Norman

Departments of Anaesthesia and *Medicine and Therapeutics, University of Leicester, Leicester Royal Infirmary, LE1 5WW

1 Cholinergic neurones in the basal forebrain are linked to cortical activation and arousal.

2 The present study was designed to examine the hypothesis that clinically relevant doses of halothane (0.1 to 5%) would significantly reduce depolarization-evoked acetylcholine (ACh) release from rat cortical slices.

3 ACh release was measured from rat cortical slices by a chemiluminescent technique.

4 Depolarization-evoked ACh release was inhibited significantly by halothane with an IC_{50} of 0.38%. This value equates to 0.3 MAC (the minimum alveolar concentration at which no movement occurs to a standard surgical stimulus in 50% of subjects) for the rat.

5 The potent effect of halothane on ACh release suggests that this mechanism may be a target for the action of volatile anaesthetic agents. This *in vitro* effect on ACh release is consistent with effects of halothane reported *in vivo*.

Keywords: Acetylcholine; transmitter release; anaesthetics, volatile; halothane; rat cortical slices

Introduction

Cholinergic neurones from the basal forebrain are involved in cortical arousal and paradoxical sleep (Buzsaki *et al.*, 1988; Steriade, 1993; Semba, 1991). Almost thirty years ago it was demonstrated that when rabbits were anaesthetized with increasing doses of halothane the amount of acetylcholine (ACh) measured on the surface of the brain decreased in a dose-dependent fashion (Kanai & Szerb, 1965). More recently, a reduction in release of ACh by halothane from the pontine reticular formation of anaesthetized cats has been demonstrated (Keifer *et al.*, 1994). These observations suggest that central cholinergic neurones involved in arousal may be a target for the action of the volatile anaesthetic agents.

Reduced ACh release under halothane anaesthesia may occur via inhibition of ACh synthesis, precursor uptake, or the release mechanism. Inhibition of synthesis is unlikely as halothane has been found to have no effect on the kinetic properties of the enzyme at the committing step in ACh synthesis, choline acetyltransferase (Griffiths *et al.*, 1994a; Johnson & Hartzell, 1985). Choline uptake is rate limiting to the synthesis of ACh (Jope, 1979) and halothane has been shown to inhibit this process with an IC_{50} (1.5%) close to the ED_{50} (1.25%) for the production of anaesthesia (minimum alveolar concentration, MAC) (Griffiths *et al.*, 1994b). *In vivo*, a reduction in the activity of the choline transporter could contribute to a reduced availability of ACh under anaesthesia. In addition, inhibition by relatively high concentrations of halothane (3%) of potassium-evoked ACh release from rat cortical synaptosomes has suggested a direct action of this anaesthetic on the release process (Johnson & Hartzell, 1985). However, no effect on potassium-stimulated ACh release was observed in rat cortical slices in the presence of 1.2% halothane (Bazil & Minneman, 1989b).

In this study the susceptibility of depolarization-evoked ACh release to inhibition by halothane was investigated across the clinically relevant dose-range to determine whether the *in vitro* response was similar to the previously reported *in vivo* effect (Kanai & Szerb, 1965; Keifer *et al.*, 1994).

[1] Author for correspondence.

Methods

Preparation of cortical slices

Female Wistar rats were killed humanely by a Schedule 1 method under the Animals (Scientific Procedures) Act 1986, the head removed *post mortem* and the brain placed in 10 ml of ice cold calcium-free Krebs buffer, pH 7.4 (composition in mM: NaCl 115, KCl 4.7, $MgCl_2$ 1.2, $NaHCO_3$ 25, glucose 8.8, HEPES 20). The cortex was dissected and cut into 350×350 μm slices with a McIlwain tissue chopper. The slices were then warmed to 37°C by washing three times with oxygenated calcium-free Krebs buffer at 37°C. None of the buffers used during the preparation of the slices contained added calcium.

For release experiments the suspension of slices was split into two equal aliquots. One was incubated for 10 min with oxygenated Krebs buffer and the other with oxygenated Krebs buffer that contained 65 μM phospholine, an organophosphorus anticholinesterase. Both sets of slices were washed subsequently with 10 ml of oxygenated Krebs three times to remove excess phospholine prior to the release experiments.

Experimental protocol

In all experiments unless stated, the release of choline and ACh from cortical slices were measured in Krebs buffer containing 2.0 mM $CaCl_2$ (unstimulated basal release) or the same buffer adjusted to contain 46 mM KCl (potassium-stimulated release) and 79 mM NaCl.

Following equilibration of buffers with the carrier gas (95% oxygen, 5% carbon dioxide) or carrier gas containing halothane for 10 min, 50 μl of packed slices was added to 100 μl of appropriate buffer and centrifuged immediately at 5,000 *g* in a refrigerated microfuge for 5 min. Supernatants were removed and frozen at −70°C until analysis. These samples were employed to determine background signal at time zero. For the measurement of ACh and choline release, 100 μl of packed slices were added to 200 μl of buffer and the tubes agitated in a water bath at 37°C with carrier gas or carrier gas containing halothane blown over the surface of the sample. After 30 min the tubes were centrifuged at 5,000 *g* for 5 min and supernatants removed and frozen as above for analysis later.

The wet weight of the slices from each incubation was determined.

Assay of acetylcholine

ACh release was quantified indirectly by substracting the value for the release of choline from tissue slices that had received pretreatment with phospholine to inhibit endogenous acetylcholinesterase from that measured from untreated slices in which both released choline and choline derived from the breakdown of released ACh was measured. Choline content of assay supernatants were measured by a modification of a coupled chemiluminescent assay (Israel & Lesbats, 1981; BioOrbit, 1990). Release of choline from slices was measured in the supernatant taken from phospholine (65 μM)-treated slices in which ACh remained undergraded.

The following reagents were used: 67 mM glycine buffer (pH 8.6), choline oxidase (Sigma C 5896, Sigma Chemical Company Poole, Dorset) 30 units ml^{-1}, microperoxidase (Sigma M 6756) 0.5 mg ml^{-1}, luminol (Sigma A 8511) diluted with 0.2 M Tris-HCl buffer to 0.5 mM. The assay volume was 1000 μl and consisted of 10 μl each of choline oxidase, microperoxidase and luminol, 870 μl of buffer and 100 μl of sample or choline standard. Luminescence was measured in a Perkin-Elmer LS 50 luminescence spectrophotometer fitted with a total emission accessory. A standard curve was constructed using choline chloride (Sigma C1879) for each batch of reaction mixture. The lower limit of detection was 50 picomol of choline. Release of choline and ACh is expressed as pmol mg^{-1} (wet weight) min^{-1}.

Anaesthetic apparatus

Halothane was delivered via a manifold of 10 outlets, each delivering 60 ml min^{-1} of humidified carrier gas (95% O_2/5% CO_2). The flow of carrier gas was controlled by a rotameter block and each outlet was calibrated with a bubble flow meter. Halothane was delivered at the required concentration from a calibrated vaporiser (Cyprane TEC 3) which had been calibrated using a laser refractometer (Index Instruments) by General Anaesthetic Services, Keighly, U.K. Control samples, exposed to carrier gas only and samples exposed to halothane were run in parallel by splitting the flow of carrier gas before the vaporiser in the circuit.

The aqueous concentration of halothane in buffer and the time to reach equilibrium of 10 min was confirmed by n-heptane extraction and measurement in a Perkin-Elmer 8410 gas chromatograph (Rutledge *et al.*, 1963). After a 10 min preincubation with 0.1, 0.2, 0.5, 3.0 and 5.0% halothane the aqueous concentrations were 0.037, 0.069, 0.134, 0.620 and 1.19 mM, respectively.

Phospholine was obtained from Cusi(UK). Halothane was obtained from Zeneca.

Statistical analysis

Results were compared by paired analysis of variance and Student's paired t test. A value of $P<0.05$ was considered statistically significant. The dose-response curves were analysed statistically by analysis of variance with a Bonferroni correction for multiple comparisons to a single control. The IC_{50} value was obtained by computer assisted curve fitting (non-linear regression model) using GraphPad (V2.0).

Results

Choline release from the rat cortical slice preparation, either untreated or treated with phospholine, was linear over 30 min under basal release conditions (4.7 mM K^+, Figure 1a) and on potassium-stimulation (46 mM K^+, Figure 1b).

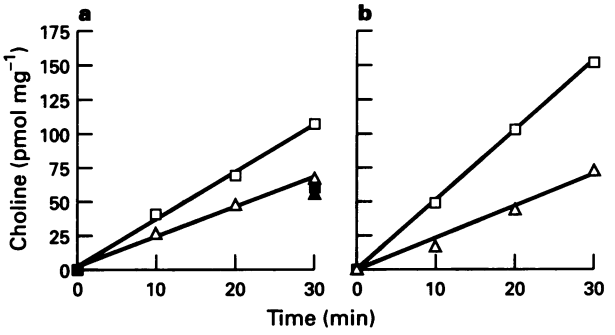

Figure 1 Time-dependence of choline release from rat cortical slices. (a) Choline release in the presence of Krebs buffer containing 4.7 mM K^+: (□) total choline (released choline plus choline derived from released ACh) from the supernatant of untreated cortical slices; (△) released choline only from the supernatant of 65 μM phospholine-treated slices. Closed symbols show the release of choline in the absence of added calcium chloride to the Krebs buffer: (▲) supernatant from slices that have been treated with phospholine and (■) supernatant from slices that were not treated with phospholine. (b) Choline release in the presence of Krebs buffer containing 46 mM K^+ Symbols as above. Range of values at 30 min stimulation with Krebs buffer containing potassium 46 mM were (□) 153 (134–169) for untreated slices and (△) 73 (70–76) for phospholine-treated slices. All points are the means of two determinations. ACh release was calculated as the difference between choline measured with and without treatment with phospholine.

Calcium-dependence

Levels of choline released from phosholine treated slices under basal conditions after 30 min in the presence and absence of calcium were essentially indistinguishable, indicating that choline release from the slice preparation under basal conditions was independent of calcium (Figure 1a and 2a).

In the absence of calcium the level of choline measured in the supernatant of slices not treated with phospholine was also indistinguishable from choline released from treated slices, indicating that there was no release of ACh in the absence of calcium from the unstimulated slices. In the presence of calcium, a larger choline concentration was measured for the untreated slices consistent with the additional release of ACh at low levels above the choline background but this did not reach statistically significant levels (Figures 1a and 2a).

Levels of choline measured after potassium stimulation of untreated slices for 30 min were significantly different when measured in the absence or presence of calcium in the buffer (Figures 1b and 2). There was no significant difference between the choline released in the presence and absence of calcium from the phospholine treated slices (Figure 2b). Subtraction of the measured choline release from the untreated and phospholine treated slices showed that significant ACh release was measured only on potassium stimulation in the presence of calcium containing buffer.

In these initial experiments, after correction for choline release, ACh release was estimated to be linear over 30 min and to be higher from the potassium-stimulated slices compared to release from slices treated with low potassium buffer (4.7 mM). The release of ACh for unstimulated and potassium-stimulated samples was calcium-dependent (Figures 1a,b and 2a,b).

Halothane

In the second series of experiments there was an approximate eight fold increase in ACh release from the potassium-evoked slices compared to unstimulated preparations. ACh release was calculated as the difference between the choline measured before and after treatment of slices with phospholine (Figure 3). ACh release from the unstimulated and potassium-stimulated slices was 0.23 pmol mg^{-1} min^{-1} (s.e.mean 0.04, $n=33$) and

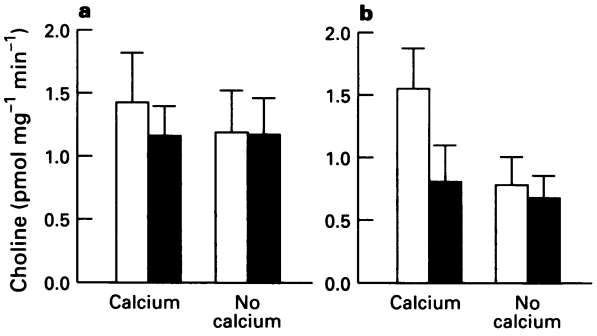

Figure 2 The effect of calcium on the rate of choline release (choline and choline derived from ACh) from cortical slices. Rate of choline release from (a) unstimulated [4.7 mM potassium containing Krebs], or (b) potassium stimulated cortical slices [46 mM potassium containing Krebs] in the presence or absence of 2.0 mM calcium chloride. Choline content of the supernatant was determined after incubation of slices for 30 min and results expressed as rate of choline release. Open columns represent total choline (choline and choline derived from ACh) released from slices not treated with phospholine; solid columns represent choline release from phospholine (65 μM)-treated slices. Results shown are mean ± s.e.mean, $n=6$ (a) and $n=4$ (b). ACh release was estimated as described in the legend of Figure 1.

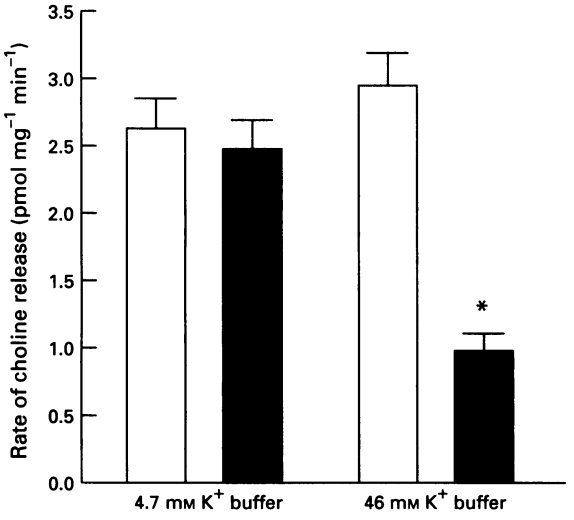

Figure 3 The rate of choline release from cortical slices. Cortical slices, either untreated (open columns) or phospholine-treated (solid columns) were stimulated with 4.7 mM potassium containing Krebs buffer (basal) or 46 mM potassium containing Krebs buffer) (stimulated). Choline content of the supernatant was determined after incubation of slices for 30 min and results expressed as rate of choline release. The results show mean ± s.e.mean, $n=33$ in each group. *Difference between phospholine-treated groups: $P<0.0001$.

1.98 pmol mg^{-1} min^{-1} (s.e.mean 0.15, $n=33$), respectively ($P<0.0001$). Although the total choline measured from basal and potassium stimulated slices not treated with phospholine was similar, the levels of choline released on potassium stimulation were significantly lower than under basal conditions. Subtraction of choline release, estimated in the presence of phospholine, from the total choline released from untreated slices revealed an increased ACh release concomitant with reduced choline release.

Halothane caused a significant reduction in potassium-evoked ACh release at all concentrations of halothane tested above 0.1% (Figure 4). Maximal inhibition of ACh release was observed at 3% halothane, above which concentration no further inhibition occurred. The IC_{50} for the inhibition of potassium-stimulated ACh release in rat cortical slices was 0.38% halothane. This equated to 0.3 MAC for the rat (White *et al.*, 1974).

There was a trend for an increase in the rate of ACh release from unstimulated samples at all concentrations of agent tested but this did not reach statistical significance (not shown).

The release of choline from the unstimulated samples was reduced significantly in the presence of 3 and 5% halothane (Figure 5).

Discussion

Potassium-stimulated acetylcholine release from the rat cortical slice preparation was inhibited in the presence of halothane. Inhibition occurred over the dose range of clinical anaesthesia, with an IC_{50} of 0.3 MAC. The potassium-stimulated release of choline from untreated slices was significantly reduced at all concentrations of halothane tested and there was a significant decrease in basal choline release at supraclinical concentrations of halothane.

Acetylcholine was measured by a modification of the chemiluminescent assay of Israel & Lesbats (1981). In the original method (Israel & Lesbats, 1981), ACh breakdown during the release phase of the assay was prevented by pretreatment of the tissue with the organophosphorus AChE inhibitor, phospholine. ACh released was then estimated by the addition of exogenous AChE in the reaction mixture. When this assay was applied to mammalian tissues there was a tendency for levels of ACh to be underestimated due to inhibition of the exogenous AChE by an endogenous inhibitor from the neural preparation (Israel & Lesbats, 1982). The identity of this inhibitor remains to be determined. Similarly, in preliminary experiments using exogenous AChE activity in the unmodified assay, we found that levels of ACh standard were underestimated in the presence of phospholine-treated brain slices (data not shown).

For this study, the assay was modified to take advantage of the endogenous AChE activity in the slice preparation. Pre incubation of part of the slice preparation with phospholine prior to release experiments prevented hydrolysis of released ACh and allowed choline release to be determined. In untreated slices, in which the endogenous AChE activity remained, released ACh was hydrolysed such that the choline measured was composed of released choline and choline derived from ACh hydrolysis. Released ACh was determined by subtraction of choline values for phospholine treated slices from those of untreated slices from the same preparation.

The rapid breakdown of ACh by AChE is a major reason why accurate measurement of released ACh has proved difficult to achieve. In this study the rate of ACh release in the presence of carrier gas only was 1.98 pmol mg^{-1} wet weight min^{-1} (s.e.mean 0.15, $n=33$). This value is consistent with that obtained by Richter (1976) who recorded 2 pmol mg^{-1} wet weight min^{-1} employing a radio enzymatic assay (Goldberg & McCaman, 1973) and by Salehmoghaddam & Collier (1976) who measured ACh release of 1.5 pmol mg^{-1} wet weight min^{-1} using the cat blood pressure assay (Brown & Feldberg, 1936). Levels of basal ACh release in 4.7 mM K^+, approximately an eighth of the release stimulated by 46 mM K^+, were also comparable to previous studies (Salehmoghaddam & Collier, 1976). The advantage of the chemiluminescent assay was that it provided an estimate of released ACh which overcame difficulties associated with the use of labelled precursors (Richter & Marchbanks, 1971).

A noticeable feature of the second series of experiments is the reduction in the choline signal in the phospholine slices exposed to potassium-stimulation. This reduction in choline level was probably caused by a stimulation of high affinity choline uptake as a result of the depolarizing conditions (Murrin & Kuhar, 1976).

Potassium-stimulated ACh release from rat cortical slices was inhibited significantly by clinically relevant doses of halothane. Reduced ACh release during anaesthesia has been implied from the 50% reduction in release seen in synaptosomes at 3% halothane concentration (Johnson & Hartzell, 1985). Our observations on ACh release from rat cortical slices

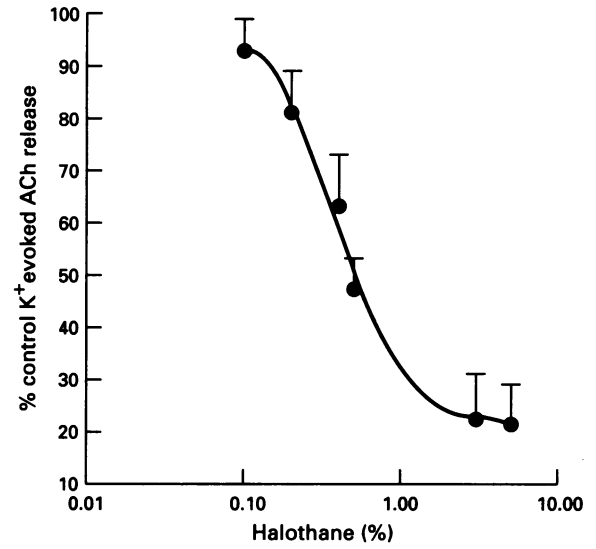

Figure 4 The effect of halothane on potassium-stimulated acetylcholine release from rat cortical slices. Slices were stimulated with 47 mM potassium containing Krebs buffer for 30 min in the presence of the required concentration of halothane and choline content of the assay supernatant measured. ACh content of the supernatant was estimated as the difference between choline content of supernatants from untreated slices and that of phospholine-treated slices and results are expressed as the rate of ACh release. Inhibition by halothane is expressed as a percentage of the control ACh release measured on the same preparation of slices. Control values were 1.98 ± 0.15 (mean ± s.e.mean, $n=33$) picomol mg^{-1} wet weight min^{-1}. Five determinations were made at each halothane concentration except at 0.4% ($n=7$) and 5.0% ($n=6$). Each point represents the mean ± s.e.mean. Anova (Bonferroni correction) P value <0.05 for 0.2% and $P<0.001$ for 0.4% to 5% versus ACh release from slices exposed to carrier gas (95% O_2/5%CO_2) only.

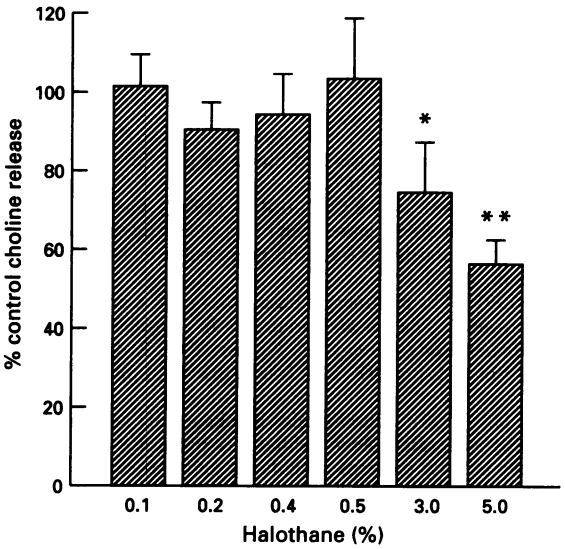

Figure 5 The effect of halothane on the release of choline from phospholine-treated rat cortical slices in Krebs buffer (4.7 mM K^+). Slices were incubated for 30 min and choline content of the supernatant was estimated. The results shown are means ± s.e.mean. There were 5 determinations in each group except 0.4% ($n=7$) and 5.0% ($n=6$) halothane. Anova (Bonferroni correction): $^*P<0.05$ and $^{**}P<0.001$.

yielded a similar reduction in release but also demonstrated that the release process was sensitive to concentrations of halothane below MAC. The IC_{50} for the reduction in release occurred at approximately 0.3 MAC. Lower concentrations of the equilibrating agent are required *in vitro* (Bazil *et al.*, 1987), but the reduction in evoked ACh release seen in these experiments suggest that the ACh release mechanism may be involved in the process of anaesthesia.

In two previous studies on rat cortical slices, no effect on ACh release was observed with halothane 1.25%, methoxyflurane 0.2% or enflurane 3% (Basil & Minneman, 1989a,b). There are a number of differences between our study and this earlier work which may explain the contrasting results. First, the earlier studies did not measure ACh release, but measured total tritium release from the preparation that had been incubated with [^{3}H]-choline. The endogenous AChE was not inhibited with an anticholinesterase and so the effect of volatile agents on total choline release rather than ACh release from the cortical slice preparation was examined. No difference in total choline was observed in the presence of anaesthetic agents (Bazil & Minneman, 1989a,b). This finding was mirrored in the present study. However, the estimation of both choline and ACh levels revealed that a reduction in the release of choline was paralleled by an increase in ACh release. This would not have been detected in the Bazil & Minneman studies.

Choline is released from the surface of neural preparations as soon as the cortex is removed from the animal (Dross & Kewitz, 1972), and the amount released varies depending on how quickly a particular neural preparation is made. Thus, the component of the choline signal from the preparation that is not derived from the hydrolysis of ACh, can vary between preparations. If only the total choline signal is measured, as in the previous studies (Bazil & Minneman, 1989a,b), any reduction in choline signal from the reduced release of ACh, may have been obscured by a choline signal derived from other sources. In this study we have measured both components of the choline signal and have demonstrated clearly a difference in the signal derived from ACh in the presence of halothane.

It is noteworthy that apart from the release of ACh, the release of a number of other important neurotransmitters from neural preparations is sensitive only to supraclinical doses of various anaesthetic agents tested (Pocock & Richards, 1988; Hirose *et al.*, 1992; El-Maghrabi & Eckenhoff, 1993). The high sensitivity of ACh release to halothane measured in this study together with the relatively high sensitivity of this process to ethanol (Carmichael & Israel, 1975) suggest that the inhibition of ACh release *in vivo* may play an important role in the depression of central activity. The rank potency for inhibition of release of a range of other transmitters by ethanol parallels that of the volatile anaesthetic agents (Carmichael & Israel, 1975; Griffiths & Norman, 1993). Moreover, the release of transmitters such as glutamate (Hirose *et al.*, 1992) and dopamine (El-Maghrabi & Eckenhoff, 1993), which have been shown to be insensitive to clinically relevant concentrations of volatile anaesthetics also require lethal doses of ethanol to inhibit this release.

In vivo, the ACh release process in the mesencephalic reticular formation in rabbits has been shown to be more sensitive to ethanol than the cortex (Erickson & Graham, 1973). Recently, a statistically significant reduction in ACh release from the pontine reticular formation of the cat has been reported (Keifer *et al.*, 1994) in the presence of 1 MAC halothane. This *in vivo* observation complements an earlier study by Kanai & Szerb (1965), in which increased dose of halothane from 1 to 1.5% caused a reduction in ACh release from the cortex of rabbits which was also linked to EEG changes. The inhibition of ACh release at similar concentrations in the present *in vitro* study suggests that halothane may have an action on ACh release.

Our study does not allow us to distinguish direct effects of the anaesthetics on the release mechanism from other activities that may affect ACh availability. We would suggest, however, that a direct action on ACh release is likely. Halothane induced inhibition of choline uptake, which is rate limiting for ACh synthesis, has been shown to occur at higher concentrations of halothane within the clinical range and also to reach a maximum inhibition of only 30% of the original activity

(Griffiths *et al.*, 1994b). Since the dose-response curve for the inhibition of choline uptake by halothane is to the right of that for ACh release, we conclude that there is likely to be a direct effect of the agent on the release mechanism which may act in concert with reduced choline uptake.

Anatomical and physiological studies have established a link between cholinergic transmission and cortical arousal (Steriade, 1993; Semba, 1991) and cholinergic neurones in the basal forebrain appear to have a crucial role in cortical arousal (Buzsaki *et al.*, 1988). The present study demonstrates that the sensitivity of potassium-evoked ACh release to halothane is within the clinically relevant anaesthetizing range of this agent. This inhibition may be responsible for the reductions in ACh release measured *in vivo* (Kanai & Szerb, 1965; Keifer *et al.*, 1994) and it appears likely that central acetylcholine release is a target for volatile anaesthetic action.

Abbreviations

ChAT, Choline acetyltransferase. MAC, minimum alveolar concentration.

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