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Anaesthetic Concentrations of Enflurane and Methoxyflurane in Rat Brain In-vivo and In-vitro

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Abstract—We measured concentrations of enflurane and methoxyflurane in brains of anaesthetized rats and established conditions for reproducing these concentrations in brain tissue in-vitro. Despite a 12-fold difference in inspired potency, brain concentrations resulting in anaesthesia were similar for both compounds. However, substantially lower concentrations in the equilibrating gas were necessary to achieve similar tissue concentrations in-vitro, probably because anaesthetic-induced respiratory depression or changes in cardiac output causes incomplete equilibration in-vivo. These studies provide direct evidence that brain concentrations associated with anaesthesia are similar for anaesthetics with different inspired potencies. They also suggest that lower concentrations in the equilibrating gas should be used in-vitro to reproduce clinically relevant tissue concentrations that are necessary to cause anaesthesia in-vivo.

General anaesthetics have been reported to affect many synaptic processes, including neurotransmitter release (Gothert 1974; Roizen et al 1975; Johnson & Hartzell 1985), neurotransmitter receptor binding (Young & Sigman 1983; Aronstam et al 1986; Dennison et al 1987), and receptor-mediated second messenger production (Okuda et al 1984). However, the molecular mechanism(s) by which these drugs act remains obscure. The inconclusive nature of this work is probably at least partly due to a failure to use clinically relevant concentrations of anaesthetics in-vitro (Trudell 1985; Rupreht & Dworacek 1986; Becker 1986; Bazil et al 1987; Scholfield 1988). Many studies used concentrations in the equilibrating gas which greatly exceed those resulting in anaesthesia in-vivo, raising the possibility that the observed changes in synaptic processes may not occur during anaesthesia. It would be very useful to know the concentrations of commonly used anaesthetics in brain during anaesthesia, so similar concentrations could be used for screening for effects on neurochemical events in-vitro. However, the concentrations of most anaesthetics in brain tissue during anaesthesia have not been determined.

We have developed a method for reproducing clinically effective concentrations of halothane in brain tissue in-vitro (Bazil et al 1987). Since different anaesthetics may act in different ways to produce unconsciousness (Metcalfe et al 1974), we wanted to establish similar methods for studying other volatile anaesthetics. We report here that two other anaesthetics with substantially different inspired potencies require similar brain concentrations for anaesthesia, and that substantially higher concentrations are attained in brain tissue in-vitro than in-vivo at similar concentrations in the equilibrating gas.

Materials and Methods

Delivery and measurement of anaesthetics

Anaesthetic levels in gas, fluids, and tissues were measured following gas chromatography (Butler & Hill 1961; Rutledge et al 1963) using a Perkin-Elmer model 8310 gas chromatograph equipped with a flame ionization detector. The six foot column was packed with 15% Apiezon L on Chromosorb W-HP (80–100 mesh). The injection temperature was 250°C. Gas samples (1 mL) were collected slowly (30 s) with a Hamilton gas-tight syringe and injected directly. Anaesthetics were extracted from buffer and tissue samples by homogenization in 0·4–2 volumes of heptane containing 0·5% hexane as an internal standard using glass centrifuge tubes or polyethylene microfuge tubes. In preliminary experiments these tubes were found not to affect anaesthetic extraction. A Brinkman PT 10/35 Polytron (Brinkmann, Westbury, NY) was used for whole brains and large brain regions, and a Kontes sonicator for brain slices. Tissue samples were centrifuged briefly before injection of 1 μL supernatant. For enflurane, the column was maintained at 80°C for 2 min, the temperature was increased to 200°C to remove heptane, and the total run time per sample was 8 min. Under these conditions, the retention times for enflurane and hexane were 0·47 and 1·37 min, respectively. For methoxyflurane determinations, the column was maintained at 60°C for 4 min following injection of sample and the temperature increased to 180°C. Total run time remained 8 min, which proved sufficient for the removal of heptane. Under these conditions, the retention times for hexane and methoxyflurane were 2·06 and 3·42, respectively. Calculations were based on integration of peak area, which was found to be linear from 1 to 1000 nmol for both anaesthetics.

Since gas samples were injected directly, no hexane internal standard was included. Measurement of these samples was therefore based directly on a standard anaesthetic sample injected separately (an external standard). For enflurane, the column was maintained at 80°C for 1·5 min, and retention time was the same as that listed above. For methoxyflurane measurements, the column was maintained at 110°C for 1·8 min, and the retention time was 1·15 min.

After evaporation of the heptane the pellet was resuspended for measurement of lipid. Lipid was measured as described by Chabrol & Charonnet (1937) and modified by

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Zollner & Kirsch (1962) using Dade total lipid standard (0·021 M oleic acid, 0·0032 M palmitic acid, and 0·0026 M stearic acid in ethanol). This method measures only unsaturated fatty acids, therefore results are best described as an index of relative lipid content rather than a quantitative determination of total lipid. This was sufficient for our purpose of normalization of anaesthetic determinations based on relative lipid content of the tissue sample. As described in our previous report (Bazil et al 1987), anaesthetic concentrations based on lipid were more consistent than those based on protein, since the amount of lipid remained fairly constant through the different tissue preparations, whereas considerable and differential amounts of protein were lost during the various preparation procedures (whole brain, slices, membrane preparations, etc.).

Anaesthetic potency and concentrations in-vivo

Male Sprague-Dawley rats were placed in individual Plexiglas chambers with an inlet for gas and an outlet for the animal's tail. One hundred percent O_2 was passed through calibrated Ohio vaporizers (model 100F, Airco, Madison, WI) for enflurane or a Pentec 2 vaporizer (Cyprane North America, Inc., Tonawanda NY) for methoxyflurane at approximately 4 L min^{-1} into the chambers. Anaesthetic concentration was monitored periodically by gas chromatography. Animals were preloaded with 5% enflurane or 2% methoxyflurane for about 5 min (until spontaneous movement stopped). Rectal thermometers were inserted and body temperature maintained at 35–38°C with heating pads. Inspired anaesthetic levels were then reduced to the desired concentration and maintained for 3 h (methoxyflurane), 1 h (enflurane) or the time indicated. Responsiveness was tested by fully clipping a haemostat to the centre of the tail. If the animal made no visible movement it was considered unresponsive. The animals were decapitated and the brain removed (< 30 s), immersed in heptane, homogenized, and anaesthetic and lipid content determined as described above.

Anaesthetic concentration in-vitro

Anaesthetics were introduced into Krebs-Ringer bicarbonate buffer (KRB; composition in mM: NaCl 120; KCl 5·5; $CaCl_2$ 2·5; $NaHCO_3$ 20; $MgCl_2$ 1·2; NaH_2PO_4 1·2; glucose 11; $CaNa_2EGTA$ 0·029) by bubbling for at least 20 min (or the time indicated) with 95% O_2/5%CO_2 that had been passed through a precalibrated vaporizer (flow = 4 L min^{-1}). All experiments were performed at 37°C. Five percent CO_2 (balance O_2) was used to equilibrate KRB in order to maintain physiological pH. This is also comparable to the 5% CO_2 present in the alveolar gas during in-vivo experiments.

To equilibrate brain tissue with anaesthetic, rats were decapitated, the brains removed, and the cerebral cortices dissected. Slices were prepared by chopping the cortices into 350×350 μm trapezoids on a McIlwain tissue slicer. Slices were dispersed using a 5 mL Gilson Pipetman, washed with KRB, drained, added to anaesthetic-equilibrated KRB at 37°C, and shaken gently in glass flasks under 95% O_2/5% CO_2 (containing the appropriate concentration of anaesthetic) for 30 min or the time indicated. The time course of incorporation of anaesthetic into tissue was determined by placing cortical slices in anaesthetic-equilibrated KRB and removing aliquots of tissue at the indicated times. Tissue was centrifuged briefly to remove buffer, sonicated in an approximately equal volume of heptane, and centrifuged again. Anaesthetic concentration was measured following gas chromatography and corrected for lipid content of the tissue as described above.

Materials

Compounds were obtained from the following sources: vanillin, Sigma; Dade total lipid standard, American Scientific Products; Ethrane brand of enflurane (Ohio Medical Products), the Emory Hospital pharmacy. Penthrane (methoxyflurane containing 0·01% w/w butylated hydroxytoluene) was generously donated by Abbott Laboratories, Chicago, Illinois.

Results

Anaesthetic concentrations in-vivo

To determine the relationship between gas concentration, brain concentration, and responsiveness, animals were exposed to different concentrations of anaesthetic. Following the preloading period, the concentration was reduced to the desired level and the animals equilibrated for 1 h (enflurane) or 3 h (methoxyflurane) to reach equilibrium (Fig. 1). At this time, animals were tested for responsiveness, killed, and brain anaesthetic content determined. The relationship between inspired anaesthetic concentrations and responsiveness is shown in Fig. 2. For enflurane, the EC50 (inspired) was about 2·8%, and the 100% anaesthetic dose (the concentration at which all animals were unresponsive) was 3·25%. For methoxyflurane, EC50 and 100% anaesthetic dose were 0·225% and 0·3%, respectively. The concentrations of anaesthetics in the brains of these same animals are shown in Fig. 3. There was a linear correlation of inspired and brain concentration over the range tested for both drugs, as was previously shown for halothane (Bazil et al 1987). Despite the 12-fold difference in inspired potency, the brain concentrations associated with anaesthesia were similar for both enflurane and methoxyflurane, being 47 and 35 nmol mg^{-1} lipid, respectively.

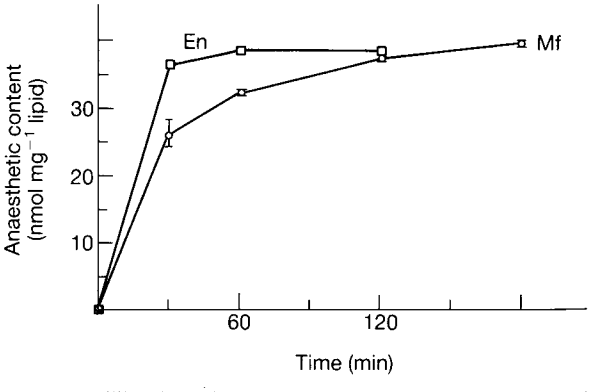

FIG. 1. Equilibration of enflurane and methoxyflurane with brain tissue in-vivo. Animals were preloaded with 5% enflurane or 2% methoxyflurane until visible movement ceased, and equilibrated with 2% enflurane or 0·5% methoxyflurane for various time periods. Brain anaesthetic content was determined as described in text. Values are mean ± s.e.m. for 3–4 determinations.

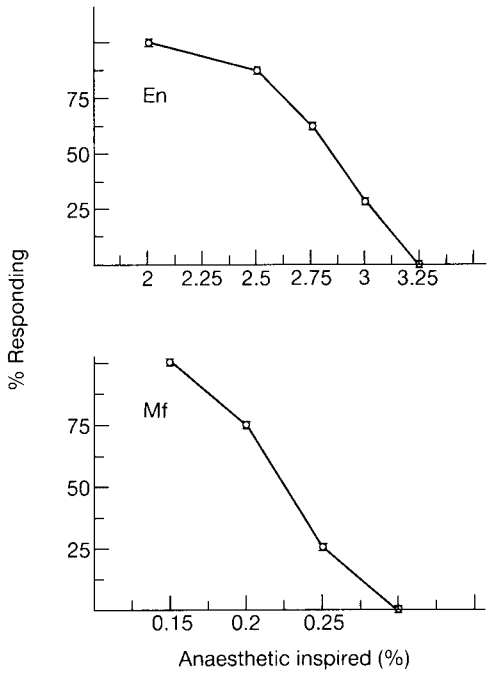

FIG. 2. Responsiveness of animals equilibrated with various concentrations of enflurane (top) or methoxyflurane (bottom). Each point represents determinations on 3–8 animals.

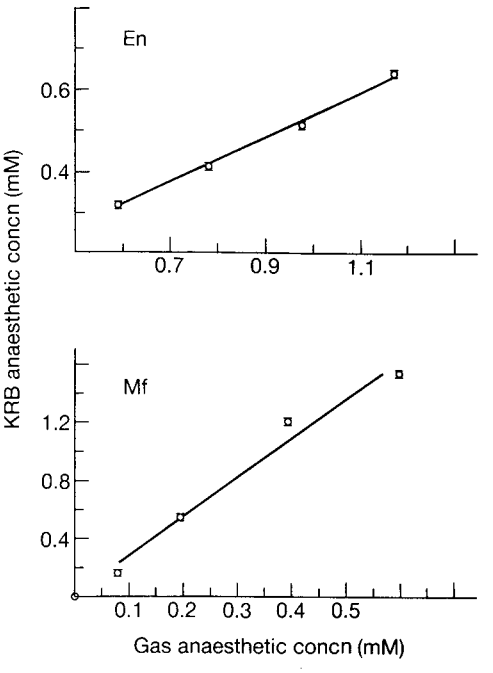

FIG. 4. Partition of enflurane (top) and methoxyflurane (bottom) from the gas into KRB. KRB was equilibrated with various concentrations of anaesthetic for 30 min, and aliquots were withdrawn. Anaesthetic content in the gas and in KRB were determined as described. Each point is the mean ± s.e.m. of three determinations.

Anaesthetic concentrations in-vitro. Enflurane and methoxyflurane both equilibrated rapidly with buffer in-vitro (less than 5 min, data not shown) as described previously for halothane (Bazil et al 1987). The relationship between the concentrations in gas and buffer is shown in Fig. 4, and was linear for both anaesthetics. The gas/buffer partition coefficients were calculated to be 0·5 ± 0·01 and 2·6 ± 0·12 for enflurane and methoxyflurane, respectively.

Both enflurane and methoxyflurane also equilibrated rapidly with brain slices in-vitro (Fig. 5). The relationship between the concentration of anaesthetics in the buffer and in brain slices is shown in Fig. 6 and was linear for both compounds. For enflurane, a 100% anaesthetic dose of 47 nmol mg^{-1} lipid was achieved by equilibrating with 1·5% in the equilibrating gas in-vitro, substantially less than the concentration required to attain this tissue concentration in-vivo (3·25%). For methoxyflurane the 100% anaesthetic

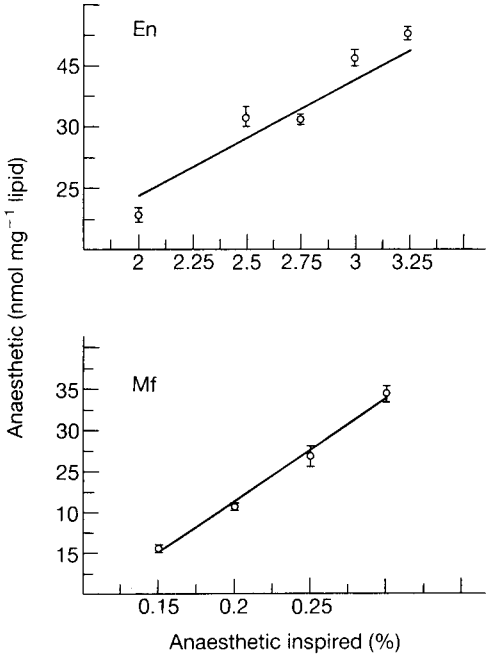

FIG. 3. Brain enflurane (top) or methoxyflurane (bottom) content in the same animals as in Fig. 2. After responsiveness was tested, each animal was decapitated and anaesthetic content determined as described. Each point is the mean ± s.e.m.

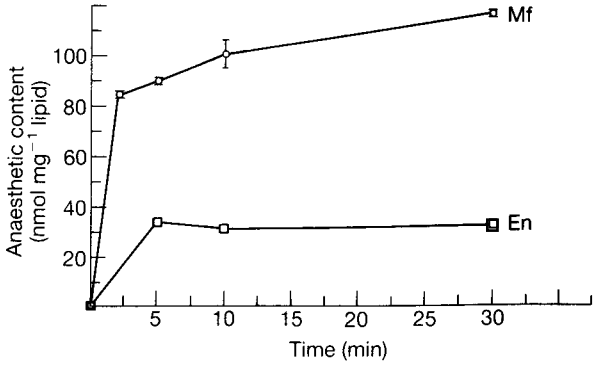

FIG. 5. Time course for equilibration of enflurane and methoxyflurane with brain slices in-vitro. Aliquots of tissue were removed at various time points and anaesthetic concentrations determined as described in text. Each point is the mean ± s.e.m. of 3–4 determinations.

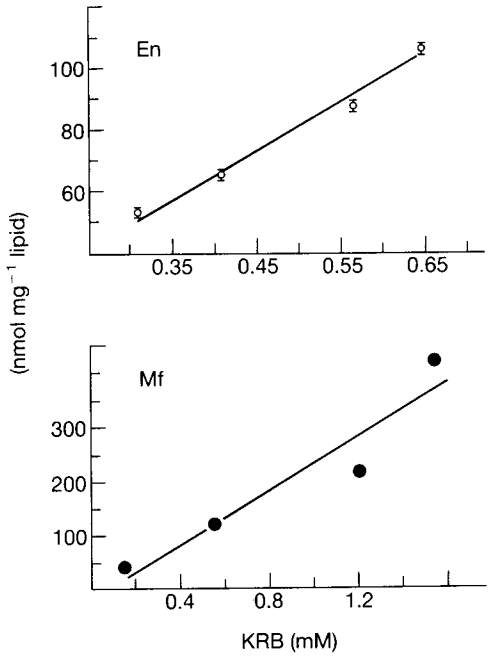

FIG. 6. Partition of enflurane (top) and methoxyflurane (bottom) from KRB into brain slices. Slices were introduced into KRB which had been pre-equilibrated with the desired concentration of anaesthetic, then incubated 30 min. Aliquots of buffer and tissue were withdrawn for determination of anaesthetic content. Each point is the mean ± s.e.m. of three determinations.

dose of 35 nmol mg^{-1} lipid was reached with 0·2% in-vitro although 0·3% was required in-vivo.

Tissue/KRB partition coefficients were estimated for these samples. Protein was determined by the method of Bradford (1976), and the lipid/protein ratio for these samples was determined to be 0·59. Protein was estimated to be one-tenth of wet weight, and the density of the tissue one g mL^{-1}. Using these approximations, the tissue/KRB partition coefficients for enflurane and methoxyflurane were determined to be approximately 10 and 13, respectively.

Discussion

The mechanism(s) by which general anaesthetics act remain unclear. One fundamental problem which, surprisingly, has not been previously addressed is the concentrations of these compounds achieved in brain during anaesthesia. Such information, and the ability to reproduce similar concentrations in-vitro, would be valuable in determining which effects of these compounds occur at concentrations associated with anaesthesia. In most previous studies, tissue concentrations have not been measured and concentrations in the equilibrating gas often greatly exceed those resulting in anaesthesia. This makes it difficult to determine the relevance of any observed effects of these compounds to the production of anaesthesia (Trudell 1985; Becker 1986; Rupreht & Dworacek 1986).

We measured brain concentrations of enflurane and methoxyflurane in rats under varying degrees of anaesthesia and reproduced similar concentrations in brain slices in-vitro. The minimum anaesthetic concentration (MAC) for methoxyflurane has been reported to be 0·22% (Eger 1974), similar to the 0·225% reported here. We are not aware of previous MAC values for enflurane in rats, however, in dogs and man enflurane MAC is 2·3–2·5 times halothane MAC (Eger 1974). We determined MAC to be 2·75% for enflurane, about 2·5 times our halothane MAC of 1·05% (Bazil et al 1987). Brain anaesthetic concentrations at which all animals are unresponsive to stimuli were determined to be 47 and 35 nmol mg^{-1} lipid for enflurane and methoxyflurane, respectively.

Our results suggest that brain concentrations during anaesthesia are relatively similar for all of the volatile agents we have studied. Despite a more than 10-fold difference in inspired potency, brain concentrations of enflurane, halothane and methoxyflurane associated with complete anaesthesia (47, 41 and 35 nmol mg^{-1} lipid, respectively) differ by only about 0·3-fold. Based on the known properties of anaesthetic agents, this is not particularly surprising, although it has not previously been demonstrated. A direct correlation between potency as an anaesthetic and lipid solubility was demonstrated over 80 years ago (Meyer 1899; Overton 1901) and predicts that, at a given inhaled concentration, the concentration in brain lipid will increase with anaesthetic potency. Therefore, brain levels achieved during anaesthesia will differ much less than the differences in inspired potency.

This observation is also consistent with theories of anaesthetic action based on physical perturbations of membrane structure, such as the critical volume hypothesis (Mullins 1954). According to this theory, anaesthetics act by dissolving in membrane lipids and physically disrupting membrane-bound structures, such as those involved with synaptic transmission. If this is true, anaesthesia would depend not on the structure of the agent but on the concentration in the lipid, which would be similar for all agents. More recent information, however, has placed doubts on this theory. Several authors have suggested that physical perturbations of membranes by anaesthetic agents either do not occur or are insignificant compared to normal physiological variance (Franks & Lieb 1979, 1981; Pang et al 1980). Our results clearly demonstrate that a fairly consistent molar concentration of anaesthetic is present in brain tissue during anaesthesia, regardless of the particular agent used, although the significance of this observation is not yet clear.

Enflurane and methoxyflurane equilibrated rapidly with buffer and brain tissue in-vitro. Gas/KRB partition coefficients were calculated to be 0·5 and 2·6 for enflurane and methoxyflurane. Somewhat higher values (0·74 and 3·8) have been reported by others (Renzi & Waud 1977); the reasons for these differences are not clear. Tissue/KRB partition coefficients were estimated to be 10 and 13 for enflurane and methoxyflurane. To our knowledge values for these partition coefficients have not previously been reported.

It is clear that substantially lower concentrations of anaesthetic in the equilibrating gas are necessary to achieve similar levels in brain tissue in-vitro than in-vivo. As we have previously suggested for halothane, this difference is probably caused by differences between inspired and alveolar anaesthetic concentrations in-vivo (Bazil et al 1987), arising largely from drug-induced respiratory depression or changes in cardiac output (Eger 1974). The largest difference was

observed with enflurane (2·2-fold higher in-vivo than in-vitro) which has a greater effect on respiration than halothane (Hirshman et al 1977). Respiratory depression by methoxyflurane is comparable to that observed with halothane (Larson et al 1969), and the in-vivo/in-vitro concentration required is comparable (1·5 vs 1·4). These observations suggest that gas concentrations of volatile anaesthetics effective in-vivo will give substantially higher tissue levels in-vitro, and underscore the importance of measuring tissue concentrations when exploring the effects of anaesthetic in-vitro.

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