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Pressure reversal of alphaxalone/alphadolone and methohexitone in tadpoles: evidence for different molecular sites for general anaesthesia

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1 Tadpoles were used to study quantitative interactions between high pressure and two intravenous anaesthetics, alphaxalone/alphadolone and methohexitone.

2 The potencies of the two agents were decreased by high pressure but to different extents. The maximum effect was seen in the pressure range 70–130 atmospheres absolute (ATA). The increases in the normobaric anaesthetizing concentration (ED_{50}) required at 100 ATA were alphaxalone/alphadolone: 405 ± 5 (s.d.)%; methohexitone: 658 ± 80 (s.d.)%.

3 For both alphaxalone/alphadalone and methohexitone, the curves obtained when the increase in ED_{50} was plotted against increasing pressure showed plateaux at pressures above 70 ATA.

4 These data support the concept of the two intravenous drugs causing general anaesthesia by the occupation of separate molecular 'sites' with different but finite capacities.

Introduction

The interactions of anaesthetics and high pressure have proved to be important in the investigations of the molecular mechanisms of anaesthesia (Smith, 1974) and of the functioning of the central nervous system under the hyperbaric conditions associated with deep sea diving (Halsey, 1982). Pressure reversal of anaesthesia was first demonstrated by Johnson and colleagues in the 1940's when it was shown that light emitted by luminous bacteria could be dimmed by anaesthetics but restored by the application of high pressure (Johnson *et al.*, 1942). This phenomenon of pressure reversal was confirmed with tadpoles by Johnson & Flagler (1950) and subsequently with newts and mice (Lever *et al.*, 1971). The critical volume hypothesis of anaesthetic action was proposed as a result of these experiments. This hypothesis states that 'anaesthesia occurs when the volume of a hydrophobic region is caused to expand beyond a critical amount by the absorption of molecules of an inert substance. If the volume of this hydrophobic region can be restored by changes of temperature or pressure, then the anaesthesia will be removed'. The assumption was made that all anaesthetics acted at the same molecular site, or at sites with the same molecular properties. The experimental evidence for this unitary assumption was that the degree of pressure reversal was the same for all those agents which had been studied at that time. Later studies with a wider range of general anaesthetics indicated that the pressure reversal data were more complicated and that the apparent molecular properties of the sites of action were not the same for all agents (Halsey *et al.*, 1978).

However, there continues to be some controversy as to whether or not the degree of pressure reversal is, or should be, independent of the anaesthetic agent itself (Halsey *et al.*, 1978; Miller & Wilson, 1978). It has been suggested that the observed differences between the anaesthetic-pressure interactions of the intravenous agents might be due to pharmacokinetic complications in the potency determination (Miller, 1980; Franks & Lieb, 1982). This paper reports results of experiments designed to investigate the quantitative interactions between pressure and two intravenous anaesthetics, alphaxalone/alphadolone and methohexitone, in the tadpole. These two anaesthetics have already been shown to have considerable differences in their pressure reversal characteristics in rats (Bailey *et al.*, 1977; Halsey *et al.*, 1978). By using tadpoles, many of the problems associated with uptake, distribution and metabolism of the agent should be avoided, and

therefore any differences between the pressure reversal characteristics of these agents would be less likely to be due simply to pharmacokinetic factors.

Methods

A small hydrostatic pressure chamber (volume 400 ml) with viewing windows was used in all experiments. This was connected via an intermediate reservoir to an oil-filled pressure pump. The reservoir prevented any oil entering the main chamber during pressurization to a maximum pressure of 467 ATA. Tadpoles were selected so that their length was 2.5 cm (range 2–3 cm): at this stage limb buds were visible but not developed and there was no air breathing. All animals used were swimming strongly. Previous experiments with tadpoles (Haw, 1981) have suggested that diet might affect anaesthetic potency and experiments in rats have shown that both age and weight can influence the potency of methohexitone (Halsey *et al.*, 1982). Although there is no direct evidence to suggest that the age or size of a tadpole critically affects responses to either anaesthesia or pressure, a standard size and diet were chosen to eliminate this possibility.

In preliminary experiments applying hydrostatic pressure in the absence of any anaesthetic treatment, four behavioural endpoints in the tadpole were identified: (a) activity associated with the external tapping of the side of the chamber, which was termed 'evoked activity'; (b) hyperactivity, which included spontaneous violent swimming; (c) tail kinking, when the muscles of the tail appeared to be continuously contracted (Mano, 1978); (d) generalized twitching. The experiments described here have used evoked activity as a method of assessing the presence or absence of anaesthesia, which was shown to be a reproducible endpoint between different observers.

Anaesthesia was studied under normobaric conditions by totally immersing the animals in a large reservoir of one of the agents in aqueous solution. The agents studied were methohexitone sodium (Brietal sodium; Lilly, U.K.) and Althesin (an iso-osmotic solution of alphaxalone 9 mg ml^{-1}, alphadolone acetate 3 mg ml^{-1}, dissolved in polyoxyethylated castor oil (Cremophor E.L.) 20%; Glaxo, U.K.). The two agents were diluted with aerated tap water to the required concentrations. The time taken to reach a stable response in the evoked activity of groups of tadpoles was established for different concentrations of each agent. From these data the effective 'induction time' for the ED_{50} could be determined. All subsequent measurements were made over periods at least twice as long as these induction times.

The pressure reversal of anaesthesia was studied by immersing the animals in a series of solutions of the agents at concentrations greater than those causing a complete loss of the evoked response and then pressurizing and decompressing. The maximum time taken for any experiment was 2.5 h. This included initial stabilization time; compression in 33 ATA stages with a 5 min hold at each pressure and decompression at a similar rate. Preliminary experiments had shown that the tadpoles would recover from these procedures if subsequently placed in fresh water, and provided the total pressure did not exceed 200 ATA.

The P_{O_2}, P_{CO_2} and pH of the solution in the chamber either with or without anaesthetic were measured before and after additional experiments. For this purpose a pH/blood gas analyser (IL 1302, Instrument Laboratory Systems) was adapted for use with water and aqueous anaesthetic solutions at room temperature. Where necessary the pH was also checked with a wide range pH meter (Model 290, Pye Unicam). Control experiments had also been carried out previously with Cremophor (the vehicle for alphaxalone/alphadolone) demonstrating that it had no effect on the behavioural response of the tadpole with or without the application of pressure (Halsey & Wardley-Smith, 1975).

A minimum of 5 groups of 5 tadpoles were used for each anaesthetic concentration and controls. The quantal data were analysed by probit analysis to determine an ED_{50} and standard deviation (s.d.). The derived probit lines were checked for parallelism and homogeneity. The results were subsequently expressed as the percentage of the control normobaric data.

Results

The tadpole responses of evoked activity at increasing pressures are shown in Figure 1. It can be seen that when exposed to pressure alone, the evoked activity does not start to diminish until almost 200 ATA pressure, and that it is completely absent by 300 ATA. By this pressure almost all the tadpoles show tail kinking, and the only movement present is a generalized random twitching. In view of the possible complications of assessing anaesthesia when some degree of pressure paralysis is present, data for pressure-anaesthetic interactions are given only for pressures up to 200 ATA.

The partial pressures of seven inhalational anaesthetics which produce a loss of evoked response in tadpoles (ED_{50}) were calculated from data obtained in earlier experiments (Halsey & Wardley-Smith, 1975) and compared with the partial pressures required to produce surgical anaesthesia in man (Quasha & Eger, 1981) (Figure 2). Regression analysis of these data indicates that $R^2 = 0.984$ after adjustment for the degrees of freedom.

The concentrations of the two intravenous agents causing a loss of evoked activity at normal pressures

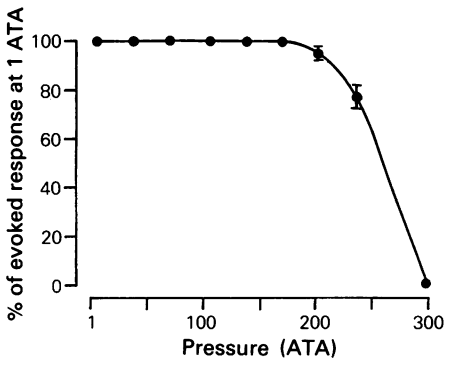

Figure 1 Evoked activity (% tadpoles responding to a standard stimulus) plotted against increasing pressures in ATA. Evoked activity was defined as tadpole swimming in response to external tapping of the chamber, and the number responding to this stimulus in each group was noted. The percentage effect is based on a total of 25 tadpoles (5 groups of 5). Error bars show s.e.mean.

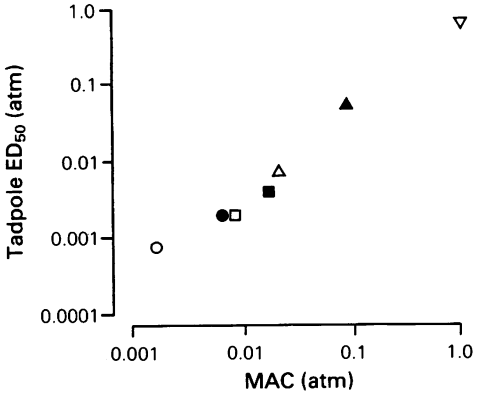

Figure 2 Plot of the partial pressures (in atmospheres) or 7 inhalational anaesthetics which produce a loss of evoked response in tadpoles against the partial pressures which produce surgical anaesthesia in man. A logarithmic scale is used to accommodate the 1,000 fold range of potencies between the agents: (○) methoxyflurane; (□) chloroform; (●) halothane; (■) enflurane; (△) diethyl ether; (▲) cyclopropane; (▽) nitrous oxide.

(ED_{50} (s.d.)) were: alphaxalone/alphadolone 1.68 (0.2) $mg\,l^{-1}$ (total steroid content); methohexitone 0.09 (0.004) $mmol\,l^{-1}$. The 'induction times' for two agents were: alphaxalone/alphadolone 1 min, methohexitone 2.5 min. The rate of loss of the behavioural response was found to be adequately described by a single exponential curve.

The pressure reversal characteristics of both agents were determined by a series of pressure experiments at different drug concentrations. These concentrations were more than sufficient to produce anaesthesia (as defined by a loss of evoked activity) in all tadpoles under normobaric conditions, but as the pressure was increased the evoked activity returned. The changes in potency were instantaneous (i.e. within the period of adjusting to pressure) and were not dependent on whether the final pressure was approached from higher or lower pressures. These data enabled dose-response curves at different pressures to be plotted, and examples of these are shown in Figure 3.

The 400 ml capacity of the pressure chamber was demonstrated to be more than sufficient to maintain the stability of the P_{O_2}, P_{CO_2} and pH over the entire period of the experiment both with and without anaesthetic (Table 1).

The complete data for the ED_{50}s of both alphaxalone/alphadolone and methohexitone at elevated pressures relative to the control normobaric values are presented in Figure 4. These results demonstrate two clear aspects of the pressure reversal characteristics of the agents. First, the degree of antagonism between pressure and the anaesthetic rose to a plateau, or a maximum, in the region of 70–130 ATA. The other striking aspect of the pressure reversal data is the difference in the magnitude of the effects for the two agents. The degree of pressure reversal of methohexitone anaesthesia is approximately twice that for alphaxalone/alphadolone anaesthesia.

Discussion

Tadpoles have been used in mechanistic studies of both anaesthesia (Dluzewski *et al.*, 1983) and of high pressure (Johnson & Flagler, 1951). Previous studies with wild tadpoles in our laboratory (Halsey & Wardley-Smith, 1975; Wardley-Smith & Halsey, 1976) suggested that there might be differences in the pressure reversal characteristics of the agents. For example, the AD_{95} (the anaesthetic dose associated with a 95% diminution of activity in a group of tadpoles) at 100 ATA varied from 150% to 250% of that at 1 ATA depending on the agent (Wardley-Smith & Halsey, 1976). However, the AD_{95} is a relatively imprecise point on the dose-response curve and the type of stimulus-response associated with the endpoint at pressure was variable in the early studies.

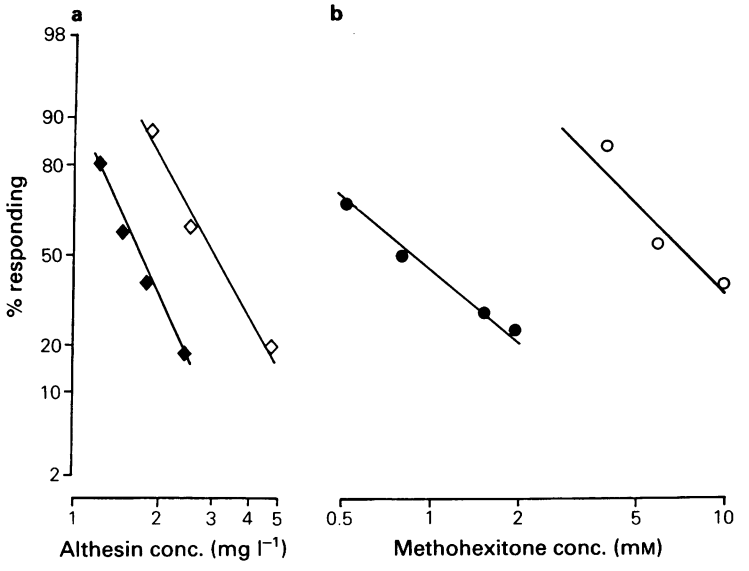

Figure 3 Some of the dose-response curves for (a) Althesin (alphaxalone/alphadolone) and (b) methohexitone on a log-probit scale. In each part of the figure the two lines are drawn through data obtained at 1 ATA (solid symbols) and 67 ATA (open symbols) respectively. The slope of the lines for each agent at 1 and 67 ATA are not significantly different.

In this study the ED_{50} has been used in preference to the AD_{95} in order that more precise estimations of the degree of pressure reversal could be made. We used evoked activity as the behavioural endpoint for the study of pressure reversal of anaesthesia, and studied principally the pressure range below 150 ATA. The subsequent analysis used the data obtained only at selected pressures (Figure 4) and it should be noted that the plateaux in the pressure reversal curves (Figure 4) occurred in a pressure range (70–130 ATA) below that which affects evoked activity in the absence of anaesthetic (see Figure 1).

We believe that the most satisfactory explanation, at present, for these data are that the two agents act at different molecular sites with different compressibilities but finite capacities, as predicted by the 'multi-site hypothesis' of general anaesthesia (Halsey *et al.*, 1978). This hypothesis was based on the rat model with steady state infusion of the intravenous anaesthetics; however, physiological limitations of the high pressure neurological syndrome (Halsey, 1982) prevented the predicted plateaux in the pressure reversal curves being unequivocally established for all agents. A mathematical analysis of the shape of the pressure reversal curves (Halsey *et al.*, 1981) indicated that the data were best fitted to an equation of the

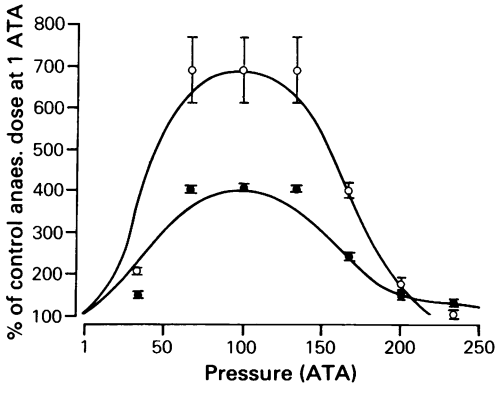

Figure 4 The ED_{50}s (± 1 s.d.) for the concentrations of alphaxalone/alphadalone (●) and methohexitone (○) required to produce a loss of evoked response expressed as a % of the control ED_{50} dose obtained at 1 ATA. The ED_{50} (s.d.) at each pressure was estimated by probit analyses of the raw data (e.g. that illustrated in Figure 3). Horizontal axis: increasing pressure in ATA.

Table 1 Biochemical variables of chamber solutions

	P_{O_2}	P_{CO_2}	pH
Before control experiment	20.8 kPa	678 Pa	7.84
After control experiment	19.8 kPa	891 Pa	7.80
Before methohexitone experiment	21.0 kPa	878 Pa	7.96
After methohexitone experiment	20.6 kPa	758 Pa	7.99

form $y = P/(a + bp + cp^2)$ which has a maximum at $\sqrt{a/c}$. The present data are also consistent with this equation. The standard deviations for the anaesthetic ED_{50}s of methohexitone were much greater than those of alphaxalone/alphadolone (see Figure 4). The coefficients of variation were also different. Similar differences were observed in previous rodent experiments (Halsey *et al.*, 1978) and one factor may be the excitant side-effects of methohexitone increasing the inherent variability of the behavioural endpoint.

However, the concept of a separate site for these anaesthetics is not the only possible explanation for the differences in pressure reversal characteristics. For example, one could consider a single site that has specific binding affinities for the two anaesthetic agents at atmospheric pressure. When high pressure is applied to this single site, it changes in conformation in a way that the binding affinities for the anaesthetic agents are decreased with respect to the one atmosphere control values. In addition, in the course of the compression of this anaesthetic site, the binding site is changed such that one of the anaesthetic agents binds particularly poorly to the binding site and therefore especially high anaesthetic concentrations are required to cause anaesthesia at high pressure. Such a binding site is different from that envisaged in the critical volume hypothesis (Lever *et al.*, 1971), and it is more complicated than a separate sites model.

Any compressed binding site model, whether single or multi-site, leads to the postulate that the binding site is not continuously compressible, but there is a pressure above which (for example, 100 atmospheres) no further alteration of the structure of the site occurs no matter how high the pressure is raised. This phenomenon could occur in a protein or lipid binding site when the molecules come to within their van de Waals' radii.

It is unlikely that these data can be explained on the basis of simple biochemical changes. First it has been suggested that the fraction of total barbiturate concentration that is uncharged will be changed during the experiment (associated, for example, with a build-up of CO_2). It is not clear what would be the effect of such a change in the reservoir solution but in any case the biochemical measurements (Table 1) argue against such phenomena. Further arguments against artifactual explanations for the data include the facts that the changes in potencies were effectively instantaneous and were not affected by whether the endpoint had been approached from a higher or lower pressure.

In addition to the theoretical discussions, it must be noted that the tadpole as an animal model for the investigations of molecular mechanisms of general anaesthesia has some disadvantages relative to an air breathing mammal. However, it does have well-defined organ systems and we have demonstrated that the relative sensitivity of its nervous system to inhalational anaesthetics correlates with that of man (Figure 2). This comparison is in terms of partial pressures of the agents which avoids the problem of differential absorption by tissues. The slope of the line relating the two effects is not unity as would be expected since loss of response to tapping the side of the chamber must occur at a lower 'depth' of anaesthesia relative to loss of response to a surgical stimulus. The tadpole has the advantage that the behavioural responses indicate that the pharmacokinetics of anaesthetic uptake follow a single exponential curve and thus approximate to a single compartment model. However, more importantly for the present study, high pressure can be applied and removed quickly without any complicating factors such as decompression sickness. This reduces the possibility of redistribution effects or agent metabolism influencing the anaesthetic potency measurements at different pressures.

The molecular interpretations of the present pressure-reversal data are consistent with the neurophysiological and neuropharmacological approaches to studying the mechanisms of these agents, which in general conclude that different anaesthetics act on different structures in neuronal membranes to produce anaesthesia. For example, the effects of alphaxalone/alphadolone and methohexitone have been compared on synaptic excitation and inhibition in the spinal cord; the results showed clear differences between the agents suggesting that each anaesthetic has an individual spectrum of neurophysiological effects which contributes to its own anaesthetic properties (Lodge & Anis, 1984). Studies in intact animals and in synaptic preparations of the olfactory cortex demonstrated that alphaxalone/alphadolone and methohexitone had synergistic, rather than additive, effects which again was interpreted as evidence against classical unitary hypotheses (Richards & White, 1981). The potassium-stimulated γ-aminobutyric acid (GABA) and D-aspartate release from cortical brain slices was inhibited by methohexitone

but not by alphaxalone/alphadolone (Minchin, 1981). There are also dissimilar influences of the barbiturates and alphaxalone/alphadolone on the responses of the lamprey reticulo-spinal neurones to GABA, glycine and L-glutamate (Cullen & Martin, 1982; 1984). Not all studies show major differences between the agents. For example, both barbiturates and alphaxalone enhanced depolarizing responses to GABA and muscimol in the rat cuneate nucleus slice with little effect on responses to glycine (Harrison & Simmonds, 1983; 1984), which has been interpreted in terms of the interactions with the $GABA_A$-receptor complex (Simmonds *et al.*, 1984).

The question that has to be asked about any study on an individual part of the nervous system is whether the particular effects observed are critical for the production of anaesthesia in the whole animal. It is thus important to have the complementary evidence of the overall anaesthetic behavioural responses which in the present experiments also reveal differences between the two agents. It is, of course, possible to speculate about different critical neuropharmacological actions of the drugs, each with different interactions with high pressure, and all contributing to the final endpoint of anaesthesia. However, such an interpretation is not yet possible for either anaesthesia or the phenomenon of pressure reversal of anaesthesia and thus the present data have only been considered in terms of the generalized concept of molecular sites.

In conclusion, the present pressure reversal data in tadpoles are in accord with those already observed in mammals and all indicate that for alphaxalone/alphadolone and methohexitone there is no universal linear relationship between their anaesthetic potencies and environmental pressure. This is in agreement with the predictions of the multi-site hypothesis (Halsey *et al.*, 1978) and provides further support for the concept of different sites of anaesthetic action with differing physical properties and with both finite capacities and compressibility.

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