EVIDENCE FOR THE ROLE OF CALCIUM IONS AND MITOCHONDRIA IN THE MAINTAINANCE OF ANAESTHESIA IN THE RAT

A. J. Sweetman and A. F. Esmail.

School of Pharmacy, Sunderland Polytechnic, Sunderland, England.

Received April 8,1975

Summary

Alphaxalone, the major component of the steroid anaesthetic, Althesin, inhibited the uptake of $^{45}Ca^{2+}$ into mitochondria isolated from rat brain. The subcellular distribution of calcium in the brain was measured after intraperitoneal injection of $^{45}Ca^{2+}$. The concentration of $^{45}Ca^{2+}$ in the brain reached a maximum after 3min, the greatest concentration being found in the mitochondrial fraction. Pre-treatment of rats with Althesin, hexobarbitone or halothane reduced the accumulation of $^{45}Ca^{2+}$ by brain mitochondrial fractions. The possible involvment of calcium ions in the mechanism of action of general anaesthetics is discussed.

Introduction

Many general anaesthetics have been shown to be inhibitors of mitochondrial respiration, and it has been suggested that the maintainance of anaesthesia could be due to a depression of ATP synthesis (1). Evidence against this hypothesis has resulted from the failure to demonstrate a change in brain ATP levels during anaesthesia (2-4). Criticism of the proposal that the mitochondrion is the primary site of action of anaesthetics has overlooked the fact that, in addition to the synthesis of ATP, a number of other respiratory chain-linked functions, including the uptake of divalent cations, have been described (5). In fact, the uptake of Ca^{2+} ions has been shown to initiate a faster rate of mitochondrial respiration than oxidative phosphorylation (6). Respiratory chain inhibitors, many of which are general anaesthetics, could therefore have a profound effect on Ca^{2+} uptake.

We have previously shown that the steroid anaesthetic, alphaxalone, a major component of Althesin, inhibits the oxidation of NAD-linked substrates in liver and brain mitochondria (7,8). The present paper shows that alphaxalone is a potent inhibitor of mitochondrial Ca^{2+} uptake *in vitro*, and demonstrates that a redistribution of $^{45}Ca^{2+}$ takes place *in vivo*, in the presence of the anaesthetics, Althesin, hexobarbitone and halothane.

Methods

Determination of $^{45}Ca^{2+}$ uptake in mitochondria isolated from rat brain. Rat brain mitochondria were isolated by the method of Clark and Nicklas (9), and

suspended at a protein concentration of 20-30mg/ml in a medium containing 0.25M-sucrose and 3.4mM-tris-HCl buffer, pH 7.4. The mitochondria (3mg protein) were incubated at 30°C in 25ml centrifuge tubes in a medium which contained, 187μmol mannitol, 62.5μmol sucrose, 250μmol potassium chloride, 25μmol tris-HCl buffer, pH 7.4, 15μmol magnesium chloride, 10μmol potassium phosphate buffer, pH 7.4, 10μmol sodium glutamate, 10 μmol sodium malate and 0.3μmol ATP. After 5min, 1.0μmol $^{45}CaCl_2$ (22.7nCi/μmol) was added and the mitochondria were incubated for a further 3min in a final volume of 3ml. The reaction was stopped by placing the tubes in an MSE High Speed 18 centrifuge at 0°C, setting the speed to maximum and applying the brake after 3min. The mitochondrial pellets were rinsed with 3ml sucrose-tris buffer and solubilized in 0.7ml of 10% (w/v) sodium deoxycholate. Aliquots (0.1ml) were added to 5ml scintillation fluid (NE 220) and the samples were counted using a Nuclear Enterprises (NE 8312) liquid scintillation counter.

Subcellular distribution of $^{45}Ca^{2+}$ *in vivo*. Wistar rats of either sex, weighing between 200-250g, were injected intraperitoneally with approximately 10μCi $^{45}CaCl_2$. The total $^{45}Ca^{2+}$ injected was 0.2μg/100g body weight. Anaesthetics were given in the following manner: Althesin, 12mg/Kg, intravenously; hexobarbitone, 90mg/Kg, intraperitoneally; halothane, 3%, from a Fluotech vapourizer. $^{45}Ca^{2+}$ was injected 30s after the righting reflex was lost and the animals were killed by cervical dislocation 5min later. The brains were removed quickly and homogenized in 15ml ice-cold medium (0.25M-sucrose and 10mM-tris-HCl, pH 7.4). The homogenate was then centrifuged, first at 1,500 x *g* for 3min, when the residue fraction was sedimented, then the post-residue fraction was centrifuged at 9,500 x *g* for 8min to yield a mitochondrial pellet and a post-mitochondrial supernatant. At each stage aliquots of the four fractions, namely, homogenate, residue, mitochondria and post-mitochondrial supernatant, were removed and homogenized in a tight-fitting glass homogenizer. Aliquots (0.5ml) of each suspension were solubilized in 2ml of 10%(w/v) sodium deoxycholate. Aliquots (1.0ml) of the resultant solutions were added to 10ml scintillation fluid and counted as described above.

Protein was determined by the method of Gornall *et al* (10).

$^{45}Ca^{2+}$ of specific activity 2.52mCi/μmol was obtained from the Radiochemical Centre, Amersham, U.K., and scintillation fluid (NE 220) from Nuclear Enterprises, Edinburgh, U.K.

Results

Table 1 shows that alphaxalone elicited a concentration-dependent depression of the uptake of $^{45}Ca^{2+}$ by mitochondria isolated from rat brain; 50% inhibition required 130μM alphaxalone.

Preliminary *in vivo* experiments showed that the injection of $^{45}Ca^{2+}$

Table 1

Effect of alphaxalone on $^{45}Ca^{2+}$ uptake into rat brain mitochondria in vitro

Additions	$^{45}Ca^{2+}$ uptake (nmol/3min)
Control	160 ± 4
ATP	927 ± 33
ATP + alphaxalone (65μM)	648 ± 24
ATP + alphaxalone (130μM)	458 ± 30
ATP + alphaxalone (330μM)	296 ± 6

$^{45}Ca^{2+}$ uptake was measured as described in the methods section, except that ATP, sodium glutamate and sodium malate were omitted from the controls. Alphaxalone was added at the beginning of each incubation at the concentrations shown. The values are the mean and standard error of the mean of the results obtained from four different preparations.

produced a rapid rise in the amount of $^{45}Ca^{2+}$ found in rat brain homogenates. The rise reached a maximum level after 3min; this maximum level was maintained for a further 5min. It was therefore decided to determine the subcellular distribution of $^{45}Ca^{2+}$ 5min after injection of the radioisotope. In a group of ten control animals, about 70% of the $^{45}Ca^{2+}$ in the brain was found in the mitochondrial fraction; the residue and post-mitochondrial fractions contained 19% and 11% respectively. Table 2 shows that the mitochondria concentrated $^{45}Ca^{2+}$, on a protein basis, to levels that were more than three times greater than those of the unfractionated homogenate. The post-mitochondrial fraction had only half the activity of the original homogenate. Carafoli (11) obtained similar results with rat liver mitochondria.

Pre-treatment with Althesin, hexobarbitone or halothane produced a marked change in the distribution of $^{45}Ca^{2+}$ in the brain compared with control experiments in the absence of the anaesthetics (Table 2). The mitochondrial fraction contained less $^{45}Ca^{2+}$ ($P = < 0.0005$ for Althesin and hexobarbitone,

Table 2

Effect of Althesin, hexobarbitone and halothane on $^{45}Ca^{2+}$ distribution in subcellular fractions of rat brain in vivo

Fraction	Relative content of $^{45}Ca^{2+}$ (Homogenate = 1.00)			
	Control	Althesin (12mg/Kg,i.v.)	Hexobarbitone (90mg/Kg,i.p.)	Halothane (3%,in O_2)
Residue	0.90 ± 0.05	0.73 ± 0.1	0.66 ± 0.09	0.69 ± 0.12
Mitochondria	3.17 ± 0.19	1.59 ± 0.11	1.49 ± 0.19	2.45 ± 0.17
Post-mitochondrial supernatant	0.49 ± 0.04	0.59 ± 0.2	0.60 ± 0.07	0.62 ± 0.14

The experiment was carried out as described in the methods section. The relative content of the fractions was calculated using the figures for $^{45}Ca^{2+}$ incorporated per mg protein in each fraction. The calcium content of the fractions was then related to that of the homogenate, which was arbitrarily set at a value of 1.00. The values are the mean and standard error of the mean of ten experiments in the case of the controls, Althesin and hexobarbitone, and six experiments using halothane.

and $P = < 0.01$ for halothane) than the controls. The anaesthetics did not affect the distribution of protein in the fractions, and the distribution of cytochrome oxidase, a mitochondrial marker enzyme, remained the same. Control experiments with bemigride, a central nervous system stimulant, showed that there was no significant change in the distribution of $^{45}Ca^{2+}$ in any of the fractions. The results therefore indicate that brain mitochondria isolated from rats treated with anaesthetics have a reduced ability to accumulate $^{45}Ca^{2+}$ in vivo. This finding is emphasized by a consideration of the ratios of $^{45}Ca^{2+}$ taken up by the mitochondrial fraction to that taken up by the post-mitochondrial fraction (Table 3). The anaesthetics brought about a marked fall in the ratio which was significant at the $P = < 0.0005$ level for Althesin ($n = 10$) and hexobarbitone ($n = 10$), and at the $P = < 0.01$ level for halothane ($n = 6$).

Table 3

Effect of Althesin, hexobarbitone and halothane on the ratio for the distribution of $^{45}Ca^{2+}$ between mitochondrial and post-mitochondrial fractions in rat brain in vivo

Treatment	Relative content of $^{45}Ca^{2+}$ (mitochondrial fraction/post-mitochondrial fraction)
Control	6.78 ± 0.76
Althesin	2.79 ± 0.87**
Hexobarbitone	2.29 ± 0.74**
Halothane	3.95 ± 0.58*

The experiment was carried out as described in the legend to Table 2. Values are the mean and standard error of the mean of experiments using ten animals in the case of Althesin and hexobarbitone and six for halothane.
**Significantly different from controls at $P = < 0.0005$.
* Significantly different from controls at $P = < 0.01$.

Discussion

The results presented in this paper show that the general anaesthetics, Althesin, hexobarbitone and halothane, cause a redistribution of $^{45}Ca^{2+}$ *in vivo* by decreasing the uptake of $^{45}Ca^{2+}$ into the mitochondrial fraction. This observation is correlated with the ability of the compounds to prevent mitochondrial Ca^{2+} uptake *in vitro*, which is brought about by a specific interaction with the mitochondrial respiratory chain.

Mitochondria have been shown to be important in maintaining the Ca^{2+} concentration in the cytoplasm at a low level (12). Any interference with the process of Ca^{2+} uptake by the mitochondria would result in an increase in the cytoplasmic Ca^{2+} levels, which would in turn lead to a modification of those membrane and enzyme systems in which Ca^{2+} has a regulatory role. According to Krnjević (13), two effects resulting from an increase in internal Ca^{2+} are of particular importance in anaesthesia. First, an increase in K^+ permeability

at the neuronal membrane, which would lead to a reduction in excitability. This effect would specifically antagonize the post-synaptic excitatory effect of acetylcholine, which acts by decreasing K^+ permeability, on cortical neurones (14). Second, internal Ca^{2+} has been shown to modify the spontaneous release of neurotransmitter substances from pre-synaptic sites (15-18).

The present findings constitute the first demonstration that there is a redistribution of Ca^{2+} during anaesthesia in vivo. Whether the redistribution is responsible for the induction and maintainance of the anaesthetic state remains to be established, however, the mitochondrion should be reconsidered as the primary site of action of general anaesthetics.

Acknowledgement

We wish to thank Glaxo Laboratories Ltd., Greenford, U.K. for their generous gifts of Althesin and alphaxalone.

References

1. Cohen, P.J. (1973) Anesthesiology 39, 153-164.
2. Nilsson, L. and Siesjo, B.K. (1971) Acta physiol Scand. 80, 235-248.
3. MacMillan, V. and Siesjo, B.K. (1973) J. Neurochem. 20, 1669-1681.
4. Fink, B.R. (1972) Handb. Exp. Pharm. 30, 387-408.
5. Lehninger, A.L. (1970) Biochem. J. 119, 129-138.
6. Chance, B., Mela, L. and Harris, E.J. (1968) Fed. Proc. 27, 902-906.
7. Esmail, A.F., Smith, H. and Sweetman, A.J. (1974) Biochem. Pharmac. 23, 3299-3304.
8. Smith, H., Sweetman, A.J. and Esmail, A.F. (1974) in Hasley, M.J., Millar, R.A. and Sutton, J.A., Molecular Mechanisms in General Anaesthesia, Churchill Livingstone, London, p. 192-208.
9. Clark, J.B. and Nicklas, W.J. (1970) J. biol Chem. 245, 4724-4731.
10. Gornall, A.G., Bardawill, C.J. and David, M.M. (1949) J. biol Chem. 177, 751.
11. Carafoli, E. (1967) J. Gen. Physiol. 50, 1849-1864.
12. Borle, A.B. (1973) Fed. Proc. 32, 1944-1950.
13. Krnjević, K. (1974) in Hasley, M.J., Millar, R.A. and Sutton, J.A., Molecular Mechanisms in General Anaesthesia, Churchill Livingstone, London, p. 65-89.
14. Krnjević, K., Pumain, R. and Renaud, L. (1971) J. Physiol. 215, 247-268.
15. Douglass, W.W. (1968) Br. J. Pharmac. 34, 451-474.
16. Kusano, K. (1970) J. Neurobiol. 1, 435-457.
17. Miledi, R. (1973) Proc. Roy. Soc. B 183, 421-425.
18. Alnaes, E., Meiri, U., Rahamimoff, H. and Rahamimoff, R. (1974) J. Physiol. 241, 30-31P.