A POSSIBLE MECHANISM FOR THE HALOTHANE-INDUCED INHIBITION OF MITOCHONDRIAL RESPIRATION: BINDING OF ENDOGENOUS CALCIUM TO NADH DEHYDROGENASE

E. M. GRIST and H. BAUM

Department of Biochemistry, Chelsea College, Manresa Road, London SW3 6LX, UK

Received 5 September 1974

1. Introduction

It is now well established that halothane, and several other inhalational anaesthetics, inhibit electron transfer in the region of complex I of the respiratory chain furthermore, it seems likely, on the basis of a number of observations, that the site of inhibition in intact mitochondria lies in the NADH dehydrogenase [6]. The levels of halothane required for this inhibition vary, depending on the system under investigation. In intact mitochondria (isolated from rat liver or bovine heart), utilising NAD^+-linked substrates, substantial inhibition of the State 3 (ADP-dependent) respiratory rate is evident at 290–850 nmoles halothane per mg mitochondrial protein [2,5]. It has been previously noted that much higher levels of halothane (per mg mitochondrial protein) are required to inhibit NADH oxidation in submitochondrial particles [6]. Similarly, our own preliminary experiments (see fig. 1) have indicated that approximately 18 μmoles halothane per mg protein are necessary from 50% inhibition of NADH oxidation by submitochondrial particles from beef heart.

We have recently demonstrated that halothane impairs the calcium binding capacity of isolated intact rat liver mitochondria [7,8]. It therefore seemed possible that inhibition of NAD^+-linked State 3-(or uncoupler-) stimulated respiration by halothane in intact mitochondria might be caused by mobilisation of endogenous calcium from certain binding sites and its subsequent binding to the NADH dehydrogenase.

We now present evidence that gives support to this hypothesis.

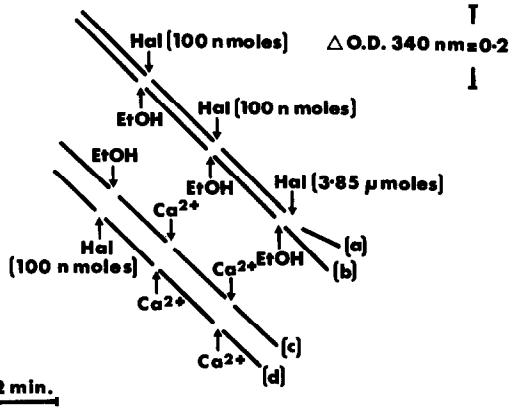

Fig. 1. The effect of halothane and calcium on the rate of NADH oxidation by submitochondrial particles. The initial reaction mixture (total vol 3 ml) contained 600 nmoles NADH and 250 μg submitochondrial particle protein in 50 mM Tris–Cl, pH 7.4. Aliquots of halothane (Hal), or an equivalent vol of ethanol (EtOH) were added as indicated. Calcium (Ca^{2+}), in two 2 nmole aliquots, was added to (c) and (d) as indicated. For convenience the figure shows changes in absorbance, rather than absolute values. The initial optical density of each sample was (a) 1.48, (b) 1.46, (c) 1.40, (d) 1.46.

2. Materials and methods

Beef heart submitochondrial particles were prepared by a modification of the method of Hansen and Smith [9]. Halothane was added as an ethanolic solution, and addition of an equal volume of absolute ethanol was made at the same time to a control sample. Calcium was added as a solution of calcium chloride, and EGTA

as a solution that has been prepared by dissolving the EGTA in potassium hydroxide solution, and adjusting the pH to 7.4 with dilute hydrochloric acid.

Oligomycin, and the uncoupler 4,5,6,7-tetrachloro-2-trifluoro–methylbenzimidazole (TTFB) were added as solutions in 95% ethanol.

All reagnets were of 'Analar' grade, or the purest grade available. Double-distilled water was used throughout.

2.1. *Assay of NADH oxidation*

(a) Aerobic oxidation was assayed by monitoring the decrease in absorbance at 340 nm, using a Perkin–Elmer spectrophotometer, model 124. The initial reaction mixture (total vol 13 ml) contained 600 nmoles NADH in 50 mM Tris–HCl buffer pH 6.4. 250 μg of submitochondrial protein were added. The reaction was followed until all of the added NADH had been oxidised.

(b) NADH-dehydrogenase was assayed by monitoring, at 415 nm, the NADH-dependent reduction of potassium ferricyanide. The initial reaction mixture (total vol 3 ml) contained 1.5 μmoles potassium ferricyanide, 2.4 μmoles NADH and 1.0 μg antimycin A in 50 mM Tris–HCl buffer pH 7.4. 52 μg submitochondrial protein were added. Further 1.5 μmole aliquots of potassium ferricyanide were added at intervals during the course of the assay, and the rate of NADH–ferricyanide reduction monitored for all levels of NADH.

3. Results

The rate of NADH oxidation (as measured by the decrease in absorbance at 340 nm) decreased gradually with decreasing residual levels of NADH. The addition of 400 nmoles of halothane per mg protein had no effect on the reaction rate, (although this level of halothane is significantly inhibitory in intact mitochondria [5]). Higher levels of halothane (18 μmole mg^{-1}) were however found to be inhibitory in these particles (fig. 1). The addition of up to 500 nmoles calcium chloride per mg protein had no effect on the rate of NADH oxidation, either in the absence of halothane, or in the presence of non-inhibitory levels (400 nmoles per mg protein) of this anaesthetic (fig. 1). However, in both the presence and absence of halothane, the addition of 800 nmoles EGTA always caused a stimulation of the rate of NADH oxidation, which was reversed by the addition of excess calcium. The EGTA stimulation in the absence of halothane also occured in the presence of oligomycin (which itself caused a 35% inhibition of the rate of NADH oxidation, reversible by uncouplers) or in the presence of an uncoupler (TTFB) alone. The effect of EGTA in the presence of uncoupler is shown in fig. 2.

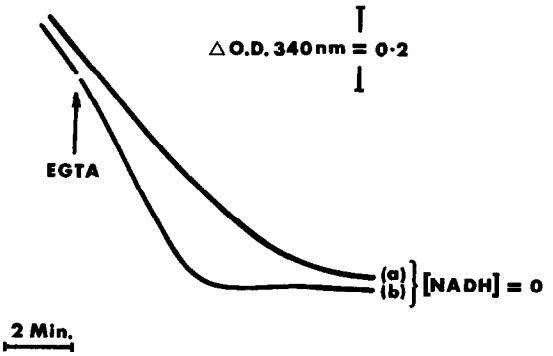

Fig. 2. The effect of EGTA on the rate of NADH oxidation by submitochondrial particles in the presence of an uncoupler. Initial reaction mixture as in fig. 1 but with the addition of 10 nmoles TTFB to each sample. (a) no further addition; (b) 800 nmoles EGTA were added as indicated.

With EGTA present, the gradual decrease in the rate of NADH oxidation, with decreasing residual levels of NADH, did not occur, and maximal activity was maintained until very low levels of NADH remained, (see fig. 2). Similar results were obtained with the NADH–ferricyanide assay system, both in terms of degree of stimulation by EGTA and shape of the progress curve.

4. Discussion

Concentrations of halothane (per mg of protein) that cause inhibition of ADP-induced State 3 (or uncoupled) respiration in intact mitochondria, oxidising NAD^+-linked substrates, have no inhibitory effect on the State 4 respiration under the same conditions; up to four times as much halothane is required before any inhibition of State 4 is seen [10]. Similarly, the lower levels of halothane have no effect on NADH oxidation by submitochondrial particles (fig. 1).

The present results show that, when exogenous calcium is added, no inhibition of NADH oxidation by these particles occurs, even in the presence of halothane (fig. 1). This observation is not, at first sight, consistent with our suggestion that it is calcium, released from mitochondrial binding sites by the action of halothane, (an agent that we have shown to decrease the calcium-binding capacity of mitochondria), that mediates the inhibition by the anaesthetic of NADH oxidation in intact mitochondria. Even if halothane did not displace calcium in submitochondrial particles, the amount of exogenous calcium added would be likely to saturate all possible sites of binding, including the NADH dehydrogenase, if this were indeed susceptible to inhibition by calcium.

In fact, however, the stimulation by EGTA of NADH oxidation routinely observed in submitochondrial particles under a variety of conditions (fig. 2), suggests that in these particles, the NADH dehydrogenase is already inhibited by calcium, presumably released from other mitochondrial sites during the sonication procedure. The previously discussed lack of effect of added calcium (with or without low levels of halothane) in submitochondrial particles therefore implies that the basic rate of NADH oxidation in such particles is one of maximal inhibition by calcium.

In intact mitochondria, therefore, the effect of halothane could be explained as follows. Respiring mitochondria will contain higher levels of endogenous calcium than are found in submitochondrial particles. However, some of this calcium will be bound to specific sites on the inner membrane, and much will also be sequestered in the matrix space. On addition of low levels of halothane some membrane-bound calcium will be relased and will then inhibit NADH oxidation, probably by binding to the NADH dehydrogenase itself. (It is unlikely that inhibition is caused by chelation of NADH by calcium, since EGTA causes stimulation of the rate of oxidation, even of relatively high levels of added NADH by submitochondrial particles). This inhibition is only rate-limiting when ADP is added, or under uncoupled conditions. The higher levels of halothane required for maximal inhibition of State 4 respiration probably reflect a general, calcium-independent effect on electron transport, such as is also seen when halothane is added at high levels to submitochondrial particles (see fig. 1). This latter effect may involve expansion of the membrane, leading to increase in separation of components of the respiratory chain; other mechanisms have also been suggested [2].

The actual extent of inhibition of NAD^+-linked respiration by the proposed calcium-dependent process would clearly depend on the steady-state concentration of NADH in contact with the dehydrogenase. As mentioned previously, and as can be seen in fig. 2, the addition of EGTA to particles alters the shape of the reaction progress curve, so that the effective degree of stimulation is much greater at lower levels of substrate. The precise characteristics of the apparent change in the kinetics of NADH dehydrogenase in the presence of calcium remains to be determined; a preliminary analysis has shown them to be rather complex. It is clear however that the effect is not merely attributable to an interference with the degree of coupling of the particles, since EGTA stimulation of NADH oxidation is seen in the presence of oligomycin and/or in the presence of uncoupler. It may well be that the change of the kinetic parameters of NADH dehydrogenase in the presence of calcium is analogous to the effect already described for α-glycerophosphate dehydrogenase by Hansford and Chappell [11]; although the change brought about by calcium in the latter case is in the opposite, (stimulatory), direction.

The binding of calcium to membranes is increased by barbiturates [12], and barbiturates such as amytal are well-known inhibitors of the span of the respiratory chain that we have now shown to be inhibited by bound calcium. It therefore seemed possible that the effect of amytal is simply the result of an increased affinity of binding of calcium at the calcium-sensitive site. However, preliminary experiments have indicated that inhibition by amytal of NADH oxidation in submitochondrial particles is not reversed by EGTA. On the contrary, EGTA stimulated respiration appears to be significantly more sensitive to amytal than is the control rate; as though, in particles, it is only in the presence of EGTA that the amytal-sensitive site maximally rate-limiting.

Acknowledgement

EG was in receipt of a Science Research Council Research Studentship.

References

[1] Miller, R. N. and Hunter, F. E. (1970) Molec. Pharmacol. 6, 67.
[2] Harris, R. A., Munroe, J., Farmer, B., Kim, K. C. and Jenkins, P. (1971) Arch. Biochem. Biophys. 142, 435.
[3] Rosenberg, H. and Haugaard, N. (1973) Anesthesiology 39, 44.
[4] Cohen, P. J. (1973) Anesthesiology 39, 153.
[5] Hall, G. M., Kirtland, S. J. and Baum, H. (1973) Brit. J. Anaesth. 45, 1005.
[6] Harris, R. A. Personal communication.
[7] Hall, G. M., Kirtland, S. J., Grist, E. M. and Baum, H. (1973) Biochem. Soc. Trans. 1, 854.
[8] Grist, E. M. and Baum, H. In preparation.
[9] Hansen, M. and Smith, A. L. (1964) Biochim. Biophys. Acta 81, 214.
[10] Hall, G. M. Personal Communication.
[11] Hansford, R. G. and Chappell, J. B. (1967) Biochim. Biophys. Res. Commun. 27, 686.
[12] Blaustein, M. P. and Goldman, D. E. (1966) Science, 153, 429.