EFFECT OF HALOTHANE ON CALCIUM TRANSPORT IN ISOLATED HEPATIC ENDOPLASMIC RETICULUM

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SUMMARY

The effects of halothane on calcium transport by microsomes prepared from rat liver have been studied. Pre-incubation of microsomal suspensions in the presence of halothane, irreversibly inactivated ATP-dependent calcium transport. The effects were dose-dependent and time-dependent and were potentiated by acid conditions (pH 6.0). The observed in vitro effects of halothane may, by increasing cytosolic concentrations of calcium, be relevant to the hepatotoxic effects of halothane.

Halothane is regarded as a safe general anaesthetic agent, but occasionally malignant hyperthermia and acute hepatic dysfunction are associated with its use.

The case for a relationship between hepatitis and halothane exposure seems to have been proven (Sherlock, 1978). Many mechanisms have been postulated, including direct hepatotoxicity of halothane or one of its reactive metabolized products (Brown and Sipes, 1977). Another proposed mechanism is an allergic or immune response (Vergani et al., 1980), but the exact mechanism is obscure, and in individual cases halothane hepatitis is still a diagnosis of exclusion.

In malignant hyperthermia the acute changes are compatible with the proposed role of an increase in cytosolic calcium concentration (Berman et al., 1970). One mechanism postulated is that halothane uncouples calcium transport from ATP hydrolysis in the sarcoplasmic reticulum of skeletal muscle. This triggering effect of halothane may be perpetuated by a vicious cycle of events resulting from irreversible acid-induced inactivation of calcium transport in the sarcoplasmic reticulum of susceptible individuals (Diamond and Berman, 1980).

In the liver, maintenance of low intracellular calcium concentration is necessary for normal hepatic function. The endoplasmic reticulum of liver actively sequesters calcium to maintain micromolar cytoplasmic concentrations against a concentration gradient (Becker, Fiskum and Lehninger, 1980). In the present study we investigated the effect of halothane on this process. In vitro, ATP-dependent uptake of calcium by rat liver microsomes was measured by assaying calcium-45 sequestered into the microsomes, and the effect of halothane on this process was studied.

This molecular effect of halothane on calcium transport may be common to many of the observed events following exposure to halothane and may provide some of the answers to the question of halothane hepatitis.

MATERIALS AND METHODS

Healthy adult male Sprague-Dawley rats were starved overnight, stunned by sharp blows to the back of their necks and decapitated. The livers were removed and immediately placed into ice-cold homogenizing medium containing sucrose 250 mmol litre\(^{-1}\), N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) 2.5 mmol litre\(^{-1}\), ethyleneglycol-bis (\(\beta\)-aminoethly ether) N, N'-tetra-acetic acid (EGTA) 0.5 mmol litre\(^{-1}\). All further manipulations were carried out at 0–4°C. Livers were washed, sliced with scissors, passed through a Harvard tissue press, and homogenized in a glass homogenizer fitted with a Teflon pestle for five or six passes. The suspension was centrifuged at 3000 \(\times\) g for 10 min and the supernatant centrifuged for 25 min at 17 000 \(\times\) g in a refrigerated centrifuge. The supernatant was then centrifuged at 100 000 \(\times\) g for 60 min and the pellet washed and resuspended carefully in sucrose 250 mmol litre\(^{-1}\), HEPES 2.5 mmol litre\(^{-1}\) to give a final protein concentration of 5–10 mg ml\(^{-1}\). Mitochondrial contamin
ation, as determined by the succinate dehydrogenase activity method of Estabrook (1967) was less than 2%. Microsomal cytochrome c reductase activity, determined by the method of Mackler (1967) was 0.35 μmol min⁻¹ mg⁻¹ of protein, in agreement with previous quoted values.

Calcium uptake by microsomes was measured in the medium described by Moore and others (1975). This consisted of potassium chloride 100 mmol litre⁻¹, histidine-imidazole buffer 30 mmol litre⁻¹, pH 6.8, magnesium chloride 5 mmol litre⁻¹, potassium oxalate 5 mmol litre⁻¹, calcium chloride 40 μmol litre⁻¹ and ⁴⁰CaCl₂ 0.1 μCi ml⁻¹. The reaction at 37°C was initiated by the addition of ATP 5 mmol litre⁻¹ to the test-tube containing microsomes and uptake medium. Non-specific binding of calcium was determined in the absence of ATP. At fixed time intervals aliquots were filtered onto millipore filters (0.45-μm pore size), washed and the residual calcium-⁴⁰ radioactivity determined in a Beckman LS233 scintillation spectrometer after dissolving the filters in 4 ml of Instagel (Packard Instruments).

A total of 100 rat livers was used over a period of 6 months. Each freshly prepared microsomal suspension was tested and discarded if contaminated or partially denatured during preparation. The fresh microsomes were used immediately and all experiments were completed within 4 h of preparation. The calcium uptake determined during testing was used as the baseline for the halothane inactivation experiments. Nonspecific binding of calcium was determined with every manipulation of each batch of microsomes.

The effect of halothane on the ATP-dependent uptake of calcium ions was investigated by pre-incubating the microsomes in vitro in the absence of calcium chloride or calcium-⁴⁰ in media containing varying halothane concentrations as previously described by Diamond and Berman (1980). Timed pre-incubations were performed at 37°C in a narrow-gauge, thick-walled glass coil with thermostat to prevent volatilization of halothane. Samples (50 μl) were delivered from the coil at timed intervals directly into uptake medium (425 μl) kept at 0°C. Under these conditions residual calcium transport remained constant until assay (within 60 min). All calcium uptake studies were performed at 37°C and were initiated by addition of ATP 5 mmol litre⁻¹ (25 μl). All experiments were repeated four or five times in duplicate and the average values were used to plot each point. An approximate 90% confidence interval for all the points was within 8% of the mean. The first order rate constant of inactivation, k_f, was calculated by assuming the logarithmic relationship and the null hypothesis was rejected by using computer-assisted regression analysis. Protein concentration of suspensions of microsomes was determined by the Folin method (Lowry et al., 1951), using sodium deoxycholate for solubilization with bovine serum albumin as standard.

RESULTS

The time course of ATP-dependent calcium transport by hepatic microsomal suspensions at 37°C is shown in figure 1. Calcium transport rates were non-linear, being maximal within the first 2–3 min and slower thereafter. Non-specific ATP-independent binding of calcium by the microsomal suspensions was 3–4 nmol/mg protein and did not vary with time of incubation.

The effect of halothane on ATP-dependent transport of calcium by rat liver microsomes was examined. After pre-incubation with halothane, calcium transport was measured at 2 min after the reaction was initiated with ATP, to determine the effects of halothane on the more rapid initial rate of active calcium transport. Pre-incubation with halothane did not affect non-specific binding of calcium. The time-course of inactivation of ATP-dependent calcium transport by the microsomal suspensions during pre-incubation with varying concentrations of halothane is shown in figure 2. The results indicate that the halothane effect is

![Fig. 1. ATP-dependent (■) and ATP-independent (●) calcium uptake by rat liver microsomes in vitro. Calcium uptake is plotted against time after the reaction was initiated.](image-url)
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**FIG. 2.** The effect of time of pre-incubation with halothane on subsequent ATP-dependent calcium uptake. The X-axis refers to the time each aliquot was incubated with halothane. The curves represent different halothane concentrations at neutral pH (7.0).

concentration-dependent and time-dependent. The rate of inactivation of calcium transport under different conditions can be used to calculate the first order rate constant of inactivation, $k_{\text{inact}}$.

The effect of pH on the sensitivity of microsomal calcium transport to halothane inactivation was examined by repeating the experiments at pH 6.0, 6.5, and 7.0. The first order rate constant of inactivation, $k_{\text{inact}}$, of calcium transport, was calculated and plotted against concentration of halothane added to the microsomes (fig. 3). At pH 6.0 the halothane effect was enhanced and acid inactivation was additive with halothane inactivation. Analysis of variance showed the differences between pH values to be significant at the 1% level, with a mean square error term equal to 0.26. The square root of the latter term, 0.51, is the standard error averaged over all 15 points.

Removal of halothane after pre-incubation by bubbling nitrogen through the suspension for 10 min did not restore the transport activity, nor was recovery evident after exposure to atmosphere and storage of microsomes for up to 6 h at 0°C. In control experiments, bubbling of nitrogen through the suspension did not affect calcium transport.

**DISCUSSION**

An ATP-dependent, non-linear rate of calcium transport by rat liver microsomal suspensions noted in this study confirms the findings of Bygrave (1978) of active calcium transport in liver endoplasmic reticulum. We have demonstrated that halothane inactivates calcium transport by liver microsomal suspensions at 37°C and pH 7.0. This halothane effect is both dose-dependent and time-dependent and is irreversible under the conditions studied. Acid pH potentiates this halothane effect.

Increased cytoplasmic calcium concentrations have been recognized as one of the mechanisms whereby general anaesthetics exert their effects (Krnjevic, 1972). *In vivo* halothane inactivation of active calcium transport by endoplasmic reticulum could be one of the mechanisms that result in increased cytoplasmic calcium concentration. This could explain the finding by Biebuyck and Lund (1974) that halothane induces rapid glycogenolysis during perfused liver experiments.

Indirect support of the possible role played by increased cytoplasmic calcium in hepatotoxicity of halothane in susceptible individuals comes from studies using carbon tetrachloride (CCL4) as a model
for hepatotoxicity. Following *in vivo* CCl₄ exposure, active calcium transport by rat liver microsomes is rapidly inactivated (Moore, Davenport and Landon, 1976), and increased stable cytoplasmic calcium was noted by Reynolds and Moslen (1974) as a separate effect to the lipoperoxidation produced.

Proposed potentiation of the halothane effect on endoplasmic reticulum calcium transport by acid environment during anaesthesia is compatible with the finding by Benumof and others (1976) of almost zero hepatic artery perfusion during halothane anaesthesia. Increased cytoplasmic calcium, which of itself would result in intracellular acidosis (Roos and Boron, 1981), could also be responsible for halothane-dependent calcium-induced loss of respiratory control (Grist and Baum, 1975). The resultant acidosis could further increase intracellular calcium concentrations (Roos and Boron, 1981), setting the stage for a vicious cycle of events. It is proposed that inactivation of calcium transport in the hypoxic centrilobular area which is relatively acid might somehow be involved in the development of centrilobular hepatic necrosis similar to the lesions seen in man and in an animal model recently described by Cousins and others (1979), following exposure in halothane hepatitis.

Several lines of evidence suggest some type of "allergic" basis for halothane hepatitis (Vergani et al., 1980). However, these events appear to have resulted from the liver injury, and not to have caused it (Dienstag, 1980). Inactivation of the calcium pump of endoplasmic reticulum or increased cytosolic calcium may contribute to such events. For example, endoplasmic reticulum calcium ATP-ase may be in equilibrium with plasma membrane calcium ATP-ase (Geisow, 1979) and halothane-induced irreversible conformational change of calcium ATP-ase (Diamond and Berman, 1980) might cause it to become antigenically reactive. Alternatively, increased cytosolic calcium concentrations may promote membrane fusion (Dreifuss, 1975) or stimulate membrane-bound calcium-activated phospholipases (Trump et al., 1980) and thus alter the antigenic properties of the plasma membrane of the hepatocyte.

Although further studies are required to compare the effect of other anaesthetic agents, we conclude that whatever the mechanism, halothane inactivation of calcium transport by hepatic endoplasmic reticulum should be considered as an explanation of some of the findings in halothane hepatitis.
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