Heterogeneous halothane binding in the SR Ca^{2+}-ATPase

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Abstract The activity of various Ca^{2+}-ATPases is affected by volatile anesthetics, such as halothane, commonly used in clinical practice. The effect on the enzyme in skeletal muscle sarcoplasmic reticulum (SR) is biphasic, including stimulation at clinical anesthetic concentrations and subsequent inhibition at higher concentrations. We have previously proposed that the action of a volatile anesthetic on Ca^{2+}-ATPases results from its binding in the interior of the enzyme molecule [Lopez, M.M. and Kosk-Kosicka, D. (1995) J. Biol. Chem. 270, 28239–28245]. Presently, we investigated whether the anesthetic interacts directly with the skeletal muscle SR Ca^{2+}-ATPase (SERCA1) as evidenced by binding. Photoaffinity labeling with [^{14}C]halothane demonstrated that the anesthetic binds saturably to SR membranes, and that ≈80% of the binding is specific, with a K_I of 0.6 mM. The K_I value agrees well with the concentration at which halothane half-maximally activates SERCA1. SDS gel electrophoresis of labeled membranes indicates that 38–56% of [^{14}C]halothane incorporates into SERCA1, and 38–53% in lipids. Distribution of label among the three fragments produced by controlled tryptic digestion of SERCA1 suggests heterogeneous halothane binding presumably in discrete sites in the enzyme. The results provide the first direct evidence that halothane binds to SERCA1. Potentially this binding could be related to anesthetic effect on enzyme's function.

Key words: SERCA1; Halothane; Photoaffinity labeling; Tryptic digestion

1. Introduction

Among several membrane proteins whose functions are affected by volatile anesthetics in vitro are Ca^{2+}-ATPases of plasma membranes (PMCA) and intracellular sarcoplasmic/endoplasmic reticulum (SERCA) [1–8]. Previously, we have proposed that anesthetics interfere with enzymatic function by binding in non-polar sites (cavities) within the protein [1]. Our previous studies including fluorescence spectroscopy measurements suggest anesthetic binding in the Ca^{2+}-ATPase molecule that somehow influences the conformational transitions that the enzyme normally undergoes in its catalytic cycle [1]. Whether direct binding to protein is responsible, however, needed to be confirmed by a more direct method. Therefore, we have used direct photoaffinity labeling with [^{14}C]halothane of SERCA1 of skeletal muscle SR. The technique has previously been successfully used to examine binding sites in serum albumin and a multisubunit membrane protein nAChR [9–11]. The results obtained through the photochemical approach for serum albumin are in very good agreement with other reported studies, using NMR, gas chromatography, and tryptophan fluorescence quenching [12,13].

SERCA1 has been extensively studied by numerous laboratories. Tryptic fragmentation of SERCA1 has helped to establish the location of functionally important binding sites, including that which binds nucleotide in fragment B (aa 506–1001) and the phosphorylation site in fragment A_1 (aa 199–508) [14,16]. The enzyme has been cloned, expressed and extensively mutated [17–20]. Based on hydrophobicity plots, immunological localization of epitopes, chemical derivatization, and fluorescence spectroscopy measurements, substantial progress has been made in predicting its three-dimensional folding of the globular cytosolic part and the transmembrane helical segments and the spatial arrangement of functional domains (for review, see [19–21]). Thus, the enzyme appears to be a reasonable candidate for investigation of anesthetic-membrane protein interactions. In the present study we find that halothane binds to the enzyme molecule, opening the possibility of precise identification of the anesthetic binding site(s) and potentially the mechanism of interference with the normal structural changes involved in the mechanism of long-range catalytic activation upon Ca^{2+} binding and phosphorylation.

2. Materials and methods

[^{14}C]Halothane (2-bromo-2-chloro-1,1,1-[1-^{14}C]trifluorethane) (spec. act. 51.3 mCi/mmol) was obtained from New England Nuclear (Boston, MA) and diluted with unlabeled halothane to 5 mCi/mmol. Unlabeled halothane was from Halocarbon Laboratories (Hackensack, NJ). Rabbit skeletal muscle SR was prepared in the laboratory of Dr. Giuseppe Inesi at the University of Maryland at Baltimore [22].

[^{14}C]Halothane photoaffinity labeling of SR was performed at 25°C in 0.5×4 cm quartz cuvettes in a total volume of 2.0 ml as previously described for other proteins [9–11]. Briefly, 4–5 mg SR in buffer containing 10 mM MOPS, pH 7.0 and 30% sucrose, pre-equilibrated with 100% argon, were incubated either with or without unlabeled (4 mM) halothane. Upon addition of [^{14}C]halothane (at 400 μM final concentration) the samples were exposed to 254 nm light (Oriel Pencil calibration lamp at a distance of 5 mm) for 60–100 s with continuous stirring. The unbound [^{14}C]halothane was removed by repeated washing and centrifugation of SR membranes at 100 000×g for 30 min. The pellet was resuspended in MOPS/sucrose buffer and used for trypsin digestion and SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE was performed according to Laemmli [23] or Weber and Osborn [24]. 20–50 μg of protein were loaded per well. Protein bands were identified with Coomassie Blue staining. For identification and quantitation of the covalently bound [^{14}C]halothane the respective lanes containing either the undigested or trypsin-digested SR were sliced to separate protein bands, background, and lipids that migrate with the dye front. Gel slices were dissolved in 30% H_2O_2 at 60°C for 6 h, and radiolabel incorporation was determined by scintillation counting. Before slicing the stained gels were photographed, and the film was scanned to determine the amount of protein in the bands representing the undigested SERCA1 and its tryptic fragments. The data represent analysis performed for 4 preparations using SDS-

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PAGE according to Laemmli and for 2 using the Weber-Osborn method.

Controlled proteolysis of SERCA1 was performed using trypsin under standard conditions which produce well-characterized fragments A, B, A_1, and A_2 [15,16]. Briefly, unlabeled or photoaffinity-labeled membranes were exposed to trypsin at a trypsin/SR protein ratio of 0.005 or 0.020 for 10 min at 25°C in the presence of 50 mM Tris-maleate, pH 7.4 and 80 mM KCl. The reaction was stopped by addition of soybean trypsin inhibitor at 5:1 trypsin inhibitor/trypsin ratio. The membranes were solubilized in sample buffer at 50°C for 30 min before loading on the gel. To differentiate between tryptic fragments A and B (and their derivatives) SR was labeled with fluorescein 5′-isothiocyanate (FITC) which binds selectively to Lys-515 [25,26]. Unreacted label was removed by washing. FITC fluorescence in protein bands after SDS-PAGE was observed under a fluorescent lamp before the gel was stained for protein.

Determination of the K_I of halothane was performed with 70 μM [^{14}C]halothane in the presence of 0.17–6.8 mM unlabeled halothane. SR protein concentration was 0.5 mg/ml in a total volume of 2 ml. The samples were exposed for 60 s to 254 nm light, as described above. The cuvette contents were vacuum filtered through GF/B filters, and extensively washed with cold buffer. Retained label on the filters was counted by liquid scintillation. Data points represent triplicates.

Ca^{2+}-ATPase activity of unlabeled SR was followed by the colorimetric determination of P_i according to Lecocq and Inesi [27]. Activity was assayed at 25°C in a reaction mixture containing 50 mM Tris-maleate, pH 7.4, 120 mM KCl, 8 mM $MgCl_2$, 1 mM EGTA, 17 μM free Ca^{2+}, 3 mM ATP, and 10 μM ionophore A23187 [1,28]. The assay was performed in sealed polypropylene tubes and halothane was delivered to the tube from an air-tight Hamilton syringe.

Protein concentration was determined by the Bio-Rad Protein Micro-assay based on the Bradford dye-binding procedure using BSA as a standard and by UV absorbance ($\varepsilon_{280} = 1.4 \times 10^5$ M^{-1} cm^{-1}) [29].

3. Results and discussion

Photoaffinity labeling of SR with [^{14}C]halothane resulted in stable label incorporation (not removable by membrane washing).

To evaluate the specificity of [^{14}C]halothane binding to SR,

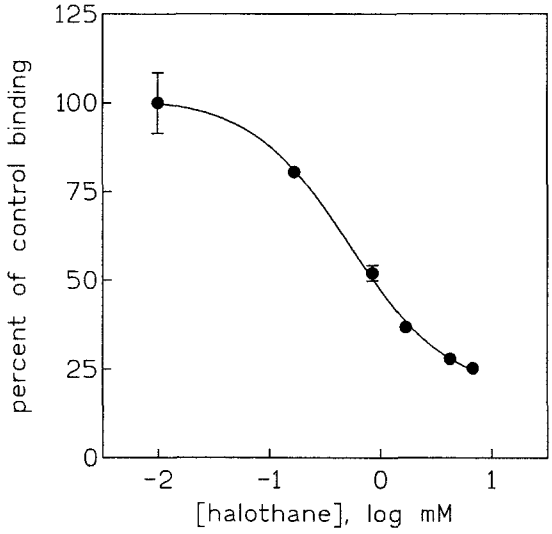

Fig. 1. Specific binding of [^{14}C]halothane to SR. SR membranes, equilibrated with increasing concentrations of unlabeled halothane, from 0.17 to 6.8 mM, were photolabeled with 70 μM [^{14}C]halothane. Control binding equals 8.9 ± 0.7 nmol/mg SR protein. The error bars indicate S.E.M. of triplicates, and are shown when their dimensions exceed those of the symbols. Line represents a non-linear least-squares fit to a sigmoid function, with the best fit found with a Hill coefficient of −1.01 and K_I of 0.6 mM.

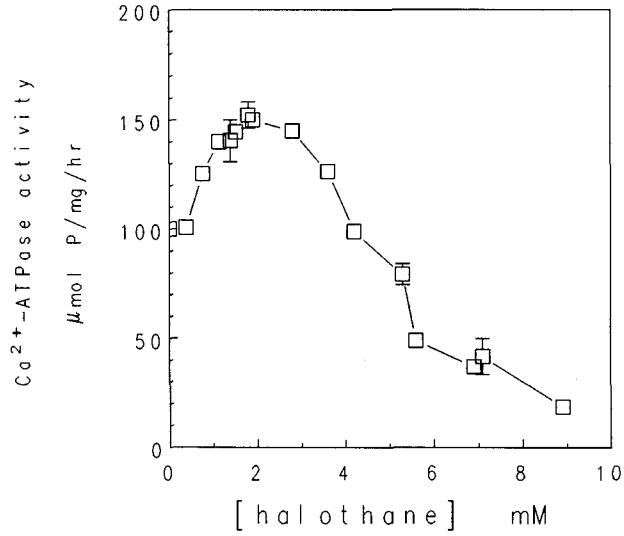

Fig. 2. Biphasic effect of halothane on SERCA1 function: activation and inhibition of Ca^{2+}-ATPase activity. Error bars shown when they exceed the dimensions of symbols.

labeling with 70 μM [^{14}C]halothane was performed in the presence of 0.17–6.8 mM unlabeled halothane (Fig. 1). These experiments reveal ≈80% inhibitable binding with a $K_I = 0.6$ mM for halothane. It has been demonstrated previously for BSA and the nAChR, that the K_I values generated using cold halothane as a competitor for [^{14}C]halothane are close to the K_D determined from Scatchard analysis of saturation binding isotherms [9,10]. This value coincides with stimulation of the Ca^{2+}-ATPase activity of SERCA1 (as shown previously in [1] and in more detail in Fig. 2). The biphasic effect explains why reports in the literature vary from activatory to inhibitory to no effect of halothane depending on the anesthetic concentration at which they were studied [4,6–8]. The fact that the halothane K_I approximates the I_{50} for stimulation of Ca^{2+}-ATPase activity suggests that the two may be related. Inhibition of the enzyme may depend on occupancy of other, lower affinity sites in the protein, or possibly may be mediated through lipids.

Under the experimental conditions (60 s UV exposure) total incorporation of label was 8.9 ± 0.7 nmol per mg protein. In calculating the stoichiometry of halothane binding to SERCA1 we considered the following facts. Of the total label incorporation 47–62% was recovered in protein, 80–90% of which bound to SERCA1 ($M_r = 110\,000$) which comprised ≈80% of the total SR protein. Thus, 3.4–5 nmol of anesthetic bind per mg SERCA1; this translates into a 0.34–0.5:1 molar anesthetic:enzyme ratio. The value represents an underestimation of unknown proportions since, in contrast to K_I values, it depends on the UV 'dose' [10]. UV exposure to complete photolysis of [^{14}C]halothane was deliberately not carried out in order to avoid cross-linking of protein and subsequent difficulty with separation.

To determine the distribution of [^{14}C]halothane into lipids and proteins, and particularly into the SERCA1 protein, we subjected the labeled SR to SDS-PAGE. After Coomassie Blue staining for protein, the entire lanes were sliced to separate protein bands, background, and lipids migrating with the dye front (see Section 2). Scintillation counting revealed 47–62% of the label incorporated into protein (38%–53% in

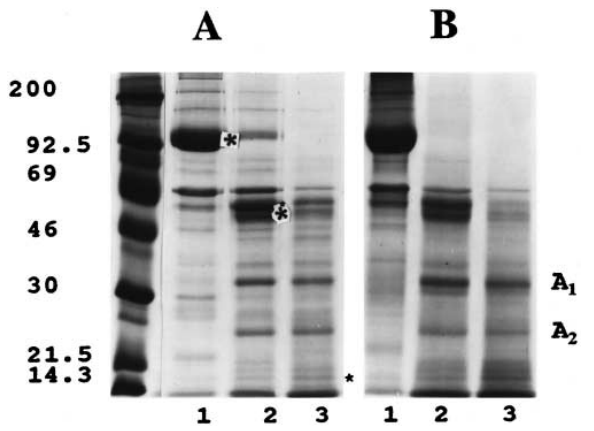

Fig. 3. Electrophoretic pattern of control (A) and [^{14}C]halothane-labeled (B) SERCA1 in undigested (lane 1) and trypsin-digested (lanes 2,3) SR vesicles. Trypsin/SR protein ratio was either 0.005 (lane 2) or 0.020 (lane 3). Asterisks denote bands (undigested enzyme, fragment B and its derivatives) in which FITC fluorescence was observed before the gel was stained with Coomassie Blue. Most fluorescence (large asterisks) was observed in undigested 110 kDa (lane 1) and fragment B (lane 2). Electrophoresis was performed on 10–15% gradient gel according to Laemmli. The M_r values shown are for Rainbow molecular weight standards from Amersham.

the lipids). Thus, 38–56% of the total labeling was recovered in SERCA1. A similar distribution of bound [^{14}C]halothane between lipids and protein was observed in nAChR [11].

To test whether the anesthetic binds preferentially in certain domains or indiscriminately all over the protein, we performed controlled tryptic digestion of SERCA1 (Fig. 3). Comparison of Fig. 3A vs. B shows that the labeled SR and its tryptic digestion patterns are comparable to those of the unlabeled SR indicating that UV exposure and [^{14}C]halothane binding do not cause any significant changes in the protein structure that would affect accessibility to the protease. Upon the initial trypsin cleavage at Arg-505 fragments, A and B were produced, followed by the appearance of fragments A_1 and A_2 when A was subsequently cleaved at Arg-198. Comparison of ^{14}C label content in excised protein bands from control and trypsin-digested SR indicates that (1) the total amounts of counts (cpm) recovered from the two lanes (undigested vs. digested SR) run in parallel were comparable within 5–15%, (2) recovery of label from protein and lipid fractions was similar, and (3) the level of labeling in the 110 kDa band diminished to zero upon increase in the trypsin to protein ratio with parallel accumulation of tryptic fragments of molecular mass lower than 20 kDa. SDS-PAGE according to Weber and Osborn allowed for separation of fragments A and B (not shown) which in the Laemmli system (that does not separate the fragments clearly) were identified by fluorescence of FITC attached to Lys-515 in the nucleotide binding domain located in fragment B (Fig. 3A).

Analyses of ^{14}C incorporation (by scintillation counting of dissolved gel slices) and protein estimation (from gel scanning) of the samples resolved by the two SDS-PAGE systems produced comparable labeling patterns (labeling to protein ratios). Fig. 3 shows an example of protein resolution in the Laemmli system. The ^{14}C label to protein ratio in the tryptic fragments (lane 2), as related to the 110 kDa protein band in the undigested SR run in parallel (lane 1) was: 0.29 in mostly A (the upper band of the two in the area of $M_r \approx 55$ kDa which shows little FITC fluorescence), 0.17 in B, 0.09 in A_1, and 0.37 in A_2. Taking into account the molecular mass, the above values translate into the following molar ratios of [^{14}C]halothane incorporation: 0.43 per 110 kDa, 0.29 per fragment A, 0.17 per fragment B, and for fragments A_1 and A_2 (including also analysis of the experiment shown in lane 3) 0.06–0.086 and 0.16–0.21, respectively. Interestingly, the sum of halothane molecules bound in fragment A_1 and A_2 is very close to the stoichiometry obtained for fragment A, and the sum of anesthetic molecules in A and B to that for the undigested 110 kDa, which is also in agreement with the stoichiometry derived from the binding experiments illustrated in Fig. 1. While these numbers need to be treated as only approximate they give a good indication of binding in various parts of the enzyme that are separated by trypsin digestion. The distribution of ^{14}C labeling does not seem to correlate to either the relative molecular mass or the number of the putative transmembrane domains in the three fragments (2 transmembrane domains in each A_1 of $M_r \approx 35\,000$ and A_2 of $M_r \approx 23\,000$ and 6 transmembrane domains in fragment B of $M_r \approx 55\,000$). In addition, while the prominent labeling of fragment A_2 does not exclude halothane binding in the nucleotide binding site it is different from the adenylate kinase for which X-ray data analysis shows the anesthetic bound only in the internal hydrophobic pocket containing the binding site for the substrate AMP [30]. The results suggest a possibility of preferential binding of volatile anesthetics in discrete sites in SERCA1 molecule, which may then produce subtle conformational changes that affect enzymatic function, and are a first step towards characterization of the sites.

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