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Halothane Does Not Alter Ca^{2+} Affinity of Troponin C

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Troponin C has been suggested as a possible target for the negative inotropic action of volatile anesthetics. This study has examined the effect of halothane on the structure and response of isolated cardiac troponin C to Ca^{2+} and the response of skinned soleus and cardiac muscle fibers to Ca^{2+}. The high-affinity Ca^{2+}-binding sites of cardiac troponin C were assessed by measurement of the change in intrinsic tyrosine fluorescence and ultraviolet circular dichroism in response to Ca^{2+} in the presence and absence of halothane. Halothane (0.9 mM, 1.4%) did not alter the 45% enhancement in intrinsic tyrosine fluorescence that occurs with saturation of the high-affinity sites or change the Ca^{2+} concentration at which half-maximal enhancement occurred. The molar ellipticity in the far ultraviolet region, a measure of the secondary structure, increased to a similar extent with addition of 10^{-6} M Ca^{2+} in the absence and presence of 1.0 mM (1.6%) halothane. The binding rate of the sulfhydryl reagent, 5,5′-dithiobis (2-nitrobenzoic acid), to troponin C in response to Ca^{2+} titration was used as a measure of the integrity of the low-affinity Ca^{2+}-binding site in troponin C in the presence and absence of 1.0 mM (1.6%) halothane. The rate of reaction was stimulated twofold, and the half maximal effect was observed at pCa 4.8 ± 0.2 in both control and halothane-treated samples. Halothane (5 mM; 7.8%) did not change the pCa/tension response of skinned soleus fibers; the data were fit to the Hill equation and yielded dissociation constants of 6.2×10^{-7} M for control and halothane-treated specimens. Cardiac skinned fibers were not altered by exposure to 1.0 mM (1.6%) halothane; Ca/tension curves were essentially identical and yielded dissociation constants of 5.9×10^{-7} M and 6.0×10^{-7} M for control and halothane-treated specimens, respectively. Halothane had no effect on the maximal tension achieved by either skinned soleus or cardiac fibers. The authors conclude from these observations that halothane does not alter the affinity of cardiac troponin C for Ca^{2+} and that the myofibrils are not an important site for the negative inotropic effect of halothane. (Key words: Anesthetics, volatile: halothane. Heart: contractile proteins; contractility; skinned fibers; troponin C.)

IT IS KNOWN that halothane depresses cardiac contractility in a dose-dependent manner.[1] Recent evidence relates the action of halothane to an alteration of Ca^{2+} homeostasis in the myocardial cell that is mediated by two main factors: a decrease in Ca^{2+} entry through the voltage-dependent Ca^{2+} channels[2,3] and a change in the release of Ca^{2+} from the sarcoplasmic reticulum, in which the total Ca^{2+} store appears to be decreased.[4,5]

It is unknown whether halothane and the other volatile anesthetics, enflurane and isoflurane, have a direct effect on the affinity and response of the contractile proteins to Ca^{2+}.[6,7] A significant decrease in myofibrillar adenosine triphosphatase activity was observed only at halothane concentrations greater (3–4%) than those used in clinical practice.[8,9] No data are available on the effect of halothane on isolated troponin C (TnC), although its involvement as a target of volatile anesthetics has been suggested by several authors. Thus, Shibata *et al.*[10] found that halothane, enflurane, and isoflurane each decreased the dynamic stiffness of Ba^{2+}-contractured papillary muscles and suggested that this effect could result from a decrease in Ca^{2+} affinity of TnC or from an alteration of voltage-dependent Ca^{2+} channels. Bosnjak *et al.* subsequently showed that the relationship between peak intracellular Ca^{2+} concentration and force was different in the presence of isoflurane compared with control, suggesting that the response of the myofibrils and perhaps of TnC to Ca^{2+} was blunted.[11] In similar observations made on intact ferret ventricular muscle, Housmans *et al.*[12] demonstrated that, at equal force, intracellular Ca^{2+} concentration was elevated in the presence of volatile anesthetics, suggesting a decreased myofibrillar responsiveness to Ca^{2+}. Most recently, Murat *et al.*,[7] using detergent-skinned cardiac fibers, observed that halothane decreased the maximal Ca^{2+}-activated tension slightly, a property that reflects Ca^{2+} binding to TnC. It also should be mentioned that Su and Kerrick[6] found that high concentrations of halo-

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thane had a small effect on mechanically disrupted bundles from rabbit hearts.

In the current study, we have used several experimental approaches to investigate the effect of halothane on the interaction of Ca^{2+} with TnC. We first examined the Ca^{2+}–troponin interaction in purified bovine cardiac troponin C (cTnC). This protein is endowed with three Ca^{2+}-binding sites, two of high and one of low affinity ($\sim 2 \times 10^7$ M^{-1} and $\sim 10^4$ M^{-1}, respectively). Ca^{2+} saturation of the latter site triggers contraction,[13] whereas binding of Ca^{2+} to the high-affinity sites results in major conformational changes. We examined the Ca^{2+}-induced change[5] in the secondary and tertiary structure of cTnC by measuring the far ultraviolet circular dichroism and the intrinsic tyrosine fluorescence in the presence and absence of 1 mM halothane. We further measured the rate of reaction of 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), a sulfhydryl reagent, with the cysteine residues of cTnC, again in the presence and absence of the anesthetic. This type of measurement focuses on the low-affinity site.[14] We then used two types of skinned fiber preparations, rabbit soleus muscle and rabbit cardiac muscle. Soleus muscle contains the same isoform of TnC as cardiac muscle.[15] We hypothesized that if there were a direct effect of halothane on TnC Ca^{2+} sensitivity, we would be able to observe it in this experimental system. Finally, experiments on skinned cardiac fibers were performed to verify the results of Murat *et al.*[7] and to determine whether there were any effects of halothane at a locus beyond TnC in the contractile process.

Materials and Methods

Cardiac troponin C was obtained from Professor C. Kay, University of Alberta, Alberta, Canada, or was prepared from fresh bovine hearts as described by Szynkiewicz *et al.*[16] The preparations were characterized by ultraviolet spectroscopy and circular dichroism.

Protein concentration was determined on the basis of an extinction coefficient (1 mg/ml, at 276 nm) of 0.3 in the presence of ethylene glycol bis (β-amino ethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA). The sulfhydryl content of cTnC, determined by means of Ellman's reagent[17] with 6 M urea, was in the range of 1.6–1.8 mol sulfhydryl group/mol cTnC. The solutions of fixed Ca^{2+} concentrations were formulated according calculations based on the program of Fabiato and Fabiato,[18] as modified by Berman‡‡; aliquots of 0.1 M $CaCl_2$ were added to solutions of 2 mM EGTA in the desired buffer. The halothane concentration in the experimental solutions was generated by taking an aliquot of buffer that was saturated with halothane and adding it to the reaction mixture. Halothane concentration was measured by ultraviolet spectroscopy.[19]

Intrinsic tyrosine fluorescence of cTnC was measured in an SLM 8000 spectrofluorometer at an emission wavelength of 300 nm, excitation wavelength of 280 nm, and a bandwidth of 4 nm. In control experiments, small aliquots of $CaCl_2$ were added to a fluorescence cuvette containing a 2-ml solution of 5 μM cTnC, 5 mM KCl, 25 mM 4-(2-hydroxyethyl)-1-piperazine sulfonic acid (HEPES), and 2 mM EGTA (*p*H 7.5) at 25° C. In the halothane experiments, 0.9 mM (1.4%) halothane was added to a solution with the same constituents as the control solution and placed in a Teflon®-sealed cuvette to maintain a constant anesthetic concentration. Aliquots of $CaCl_2$ were added with a Hamilton syringe through the Teflon® seal.

Circular dichroism experiments were performed in 10 mM 3-N-morphilino propane sulfonic acid (Mops), 25 mM KCl, and 2 mM EGTA (*p*H 7.0) at 20° C on a Jasco J500A spectropolarimeter equipped with a Jasco DP 500N data processor. The molar ellipticities (degrees · cm^2 · $dmol^{-1}$) are expressed on an amino acid residue basis (M_r = 113).

The rate of reaction of cysteine residues with DTNB was followed at 412 nm in a Cary 2200 spectrophotometer at 25° C.[14] The solutions, in 100 mM Mops containing 50 mM KCl and 2 mM EGTA at *p*H 7.0, were 5 μM in cTnC and 230 μM in DTNB. The rate of reaction was examined as a function of Ca^{2+} concentration in the presence and absence of 1 mM (1.6%) halothane; a constant concentration of halothane was maintained by sealing the cuvette with Teflon®. Pseudo-first-order rate constants were obtained by fitting the data to monoexponential equations with the Enzfitter program (R. J. Leatherbarrow, Elsevier Biosoft, Cambridge, UK).

Chemically skinned fibers were obtained from the soleus muscle and cardiac muscle of New Zealand white male adult rabbits. Muscle samples (bundles 3–5 mm in diameter and 20–30 mm long) were tied to a wooden stick and stretched to 110–120% of slack length before they were cut from the bulk of muscle tissue. Samples were skinned chemically by incubation at 0–4° C for 24 h in 10 ml "skinning solution" (5 mM K_2–EGTA/170 mM potassium propionate/2.5 mM Na_2–K_2–adenosine triphosphate/2.5 mM magnesium propionate/10 mM imidazole buffer, *p*H 7.0) by the procedure of Wood *et al.*[20] After 4 h and 8 h, the skinning solution was replaced with fresh solution.[21] After 24 h, the samples were transferred to "storage solution" with the same composition as the skinning solution, but also containing 50% (volume/volume) glycerol, and were stored at −20° C. This skinning procedure preserves the integrity of the sarcoplasmic reticulum and the architecture of the contractile proteins.[22]

To measure the tension, segments of the skinned cardiac and skeletal fibers (mean diameter, 50 μm) were dis-

‡‡ Berman M: Personal communication.

sected from the bundle and placed in a chamber containing relaxing solution that was of the same composition as the skinning solution, but with an additional 2.5 mM Na_2–adenosine triphosphate. The segments were attached between two clamps, one of them attached to a strain gauge.[23] Steady-state isometric force/*p*Ca curves were obtained by exposing the fiber sequentially to solutions of different free Ca^{2+} (from *p*Ca 6.6 to *p*Ca 4.8). All experiments were performed at room temperature (22–24° C).

We did not perform statistical analysis to determine between-group differences in the presence and absence of halothane because no differences were evident.

Results

Effect of Halothane on High-affinity Ca^{2+} Binding Sites

Ca^{2+} binding to the high-affinity sites results in an increase of the ellipticity in the far ultraviolet region and of the intrinsic tyrosine fluorescence. The enhancement of intrinsic tyrosine fluorescence was monitored at 300 nm in the presence and absence of 0.9 mM (1.4%) halothane at Ca^{2+} concentrations ranging from 10^{-8} to 10^{-3} M. The fluorescence enhancement corresponds to 45%, in accordance with data in the literature,[24] and is unaltered by halothane (fig. 1). The molar ellipticity at 222 nm decreased from −13,300 to −18,500 with the addition of 10^{-6} M Ca^{2+}, in agreement with results by Szynkiewicz *et al.*,[16] and was unchanged with further additions of the metal or 1 mM halothane (fig. 2).

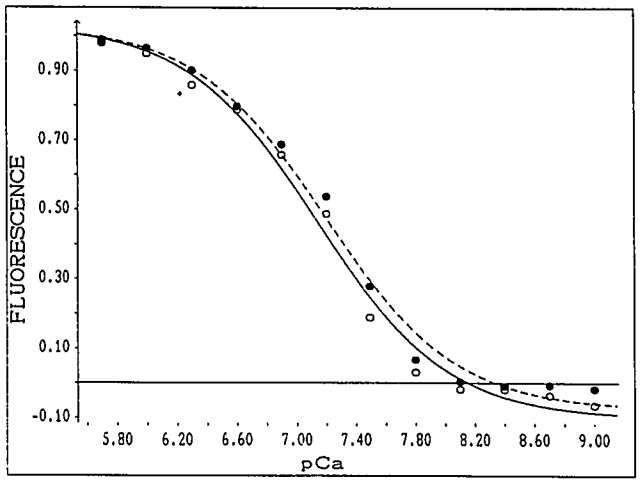

FIG. 1. Ca^{2+} titration of 5 μM cTnC in the absence (open circles) and presence (filled circles) of 0.9 mM (1.45%) halothane. Aliquots of $CaCl_2$ were added to a Teflon-capped fluorescent cuvette containing 5 mM KCl, 25 mM Hepes, and 2 mM EGTA at *p*H 7.5 and 25°C. The x-axis represents the *p*Ca (negative log of the molar concentration of Ca^{2+}), and the y-axis is the ratio of the fluorescence at each wavelength to the fluorescence maximum. Each point is the mean of five determinations.

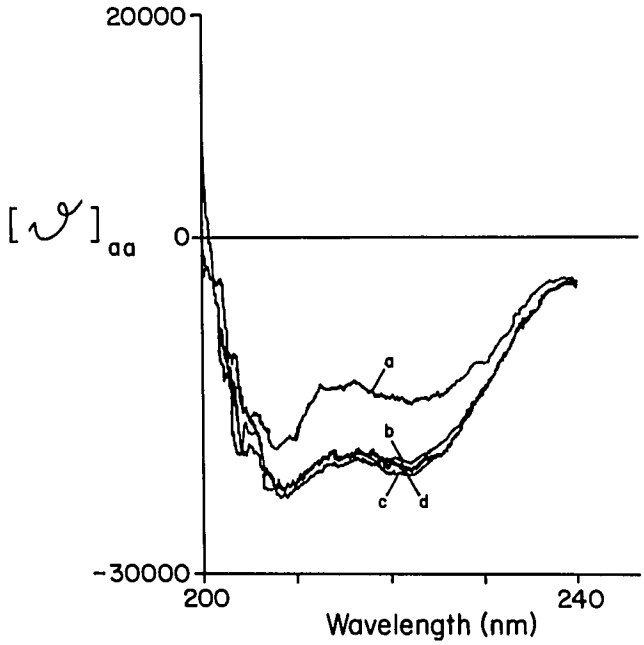

FIG. 2. CD spectra of 5 μM cTnC in the absence of calcium (a), at a *p*Ca of 6 (b), at a *p*Ca of 3 (c), and in the presence of 1 mM (1.6%) halothane at a *p*Ca of 3 (d). The x-axis is the wavelength in nanometers, and the y-axis indicates the molar ellipticity in degrees · cm^2 · $dmol^{-1}$. The experiment was performed in 10 mM Mops, 25 mM KCl, and 2 mM EGTA at *p*H 7.0 and 20°C.

Effect of Halothane on Low-affinity Ca^{2+} Binding Site

Fuchs *et al.*[14] showed that the binding rate of DTNB to the cysteine residues in positions 35 and 84 depends on the saturation of the low-affinity, regulatory Ca^{2+}-binding site of cTnC. On this basis, the rate of reaction of DTNB was measured as a function of the Ca concentration in the range 10^{-7} to 10^{-3} M in the presence and absence of 1 mM (1.6%) halothane (fig. 3). In agreement with the results of Fuchs *et al.*,[14] the reaction rate is stimulated more than twofold by Ca^{2+}. The half maximal effect is observed at *p*Ca 4.8 ± 0.2 and is not altered by halothane.

Effect of Halothane on Ca^{2+} Sensitivity and Maximal Tension of Skinned Soleus Fibers

Figure 4 demonstrates the tension response of a skinned soleus fiber to increasing concentrations of Ca^{2+} in the presence and absence of 5 mM (7.8%) halothane. This high concentration of halothane was used to amplify any effect on the soleus fiber. As seen in figure 4, control and halothane-treated soleus muscle fiber responded equally to Ca^{2+}. The data were fit to the Hill equation with the

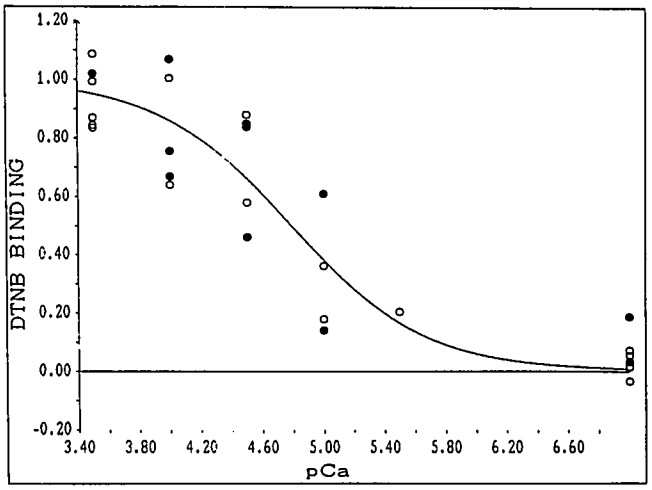

FIG. 3. The normalized rate of DTNB binding to 5 μM cTnC in the absence (open circles) and presence (filled circles) of 1 mM (1.6%) halothane at *p*H 7.0 and 20°C as a function of Ca^{2+} concentration. The experiment was performed in 100 mM Mops, 50 mM KCl, and 2 mM EGTA. The x-axis represents the *p*Ca, and the y-axis represents the normalized rate of DTNB binding. The solid line is the nonlinear regression fit of the data. The data result from two independent TnC preparations, characterized by the following asymptotic values: 4.9 $\times 10^{-2}$ and 2.1 $\times 10^{-2}$ or 3.8 $\times 10^{-2}$ and 2.1 $\times 10^{-2}$ min^{-1} at high and low Ca^{2+} concentrations, respectively.

use of the NFIT program. The derived parameters showed Hill coefficients of 5.0 (control) and 4.1 (halothane), respectively, and dissociation constants of 6.3 $\times 10^{-7}$ M and 6.4 $\times 10^{-7}$ M, respectively. These data indicate that, in soleus muscle, halothane at 5 mM has essentially no effect on the *p*Ca–tension relationship, which is related to Ca^{2+} binding to TnC.

Effect of Halothane on the Ca^{2+} Sensitivity and Maximal Tension Response of Skinned Cardiac Fibers

Figure 5A demonstrates the tension response of a skinned cardiac fiber to increasing concentrations of Ca^{2+} in the presence and absence of 1 mM (1.6%) halothane. The derived parameters are Hill coefficients of 2.8 (control) and 2.5 (halothane) and dissociation constants of 5.9 $\times 10^{-7}$ M (control) and 6.0 $\times 10^{-7}$ M (halothane). The effect of 200 μM (0.3%) halothane on the equilibrium tension at two concentrations of Ca^{2+} is shown in figure 5B. Halothane does not exert a negative inotropic effect in either situation.

Discussion

We have used both isolated bovine cTnC and two rabbit skinned fiber systems to investigate the possible role of TnC in the mechanism of anesthetic depression of contractility. Our results in these diverse systems suggest that the volatile anesthetic halothane has little, if any, effect on TnC in particular and the myofibrils in general.

Troponin C is a regulatory myofibrillar protein in both cardiac and skeletal muscle that binds Ca^{2+} and undergoes conformational changes that are transmitted to another regulatory protein, tropomyosin. In turn, tropomyosin is displaced from the actin myosin groove in the myofibril; thereafter, actin and myosin interact and initiate contraction. Cardiac troponin C contains three binding sites for Ca^{2+}, whereas skeletal TnC contains four binding sites. Cardiac troponin C has one low-affinity and two high-affinity sites. The low-affinity site is Ca^{2+} specific, whereas the high-affinity sites also bind Mg^{2+}. The affinity constants for Ca^{2+} are approximately two orders of magnitude apart ($\sim 2 \times 10^7$ M^{-1} and $\sim 10^4$ to 10^5 M^{-1}),[14,24,25] such that filling of the low-affinity site occurs only after the high-affinity ones are fully saturated. The site that is important in the initiation of contraction is the low-affinity site. The high-affinity sites, on the other hand, are saturated with Ca^{2+} and/or Mg^{2+} during both contraction and relaxation phases of the contractile cycle and, therefore, are not involved in inducing the conformational change in TnC that triggers contraction.

Isolated cTnC can be stripped of Ca^{2+}; subsequent addition of the Ca^{2+} results in a 45% enhancement of intrinsic fluorescence and a 50% increase of the ellipticity at 222 nm.[2,5] The change in both parameters is complete when the high-affinity sites are occupied with Ca^{2+} (*i.e.*, at approximately 10^{-6} M). This is evident in figure 1, which shows that the increase in fluorescence is complete at *p*Ca 5.8. Our fluorescence and circular dichroism data showed that 1 mM halothane has no effect on either the Ca^{2+} concentration dependence or the extent of the fluorescence or ellipticity increase. These results indicate

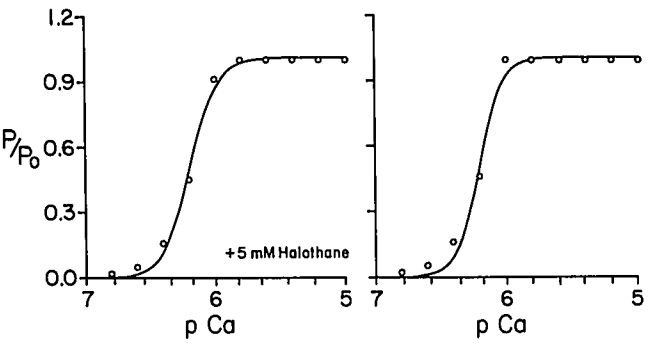

FIG. 4. The Ca^{2+}–tension curve of skinned soleus fibers in the presence and absence of 5 mM (7.8%) halothane. Each data point is the mean of three or four determinations. The experiment was performed in 5 mM K_2 EGTA, 170 mM potassium propionate, 5 mM Na_2 ATP, 2 mM magnesium propionate, and 10 mM imidazole at *p*H 7.0 and room temperature. The x-axis represents the *p*Ca, and the y-axis represents the tension (p) normalized to maximal tension (p_0).

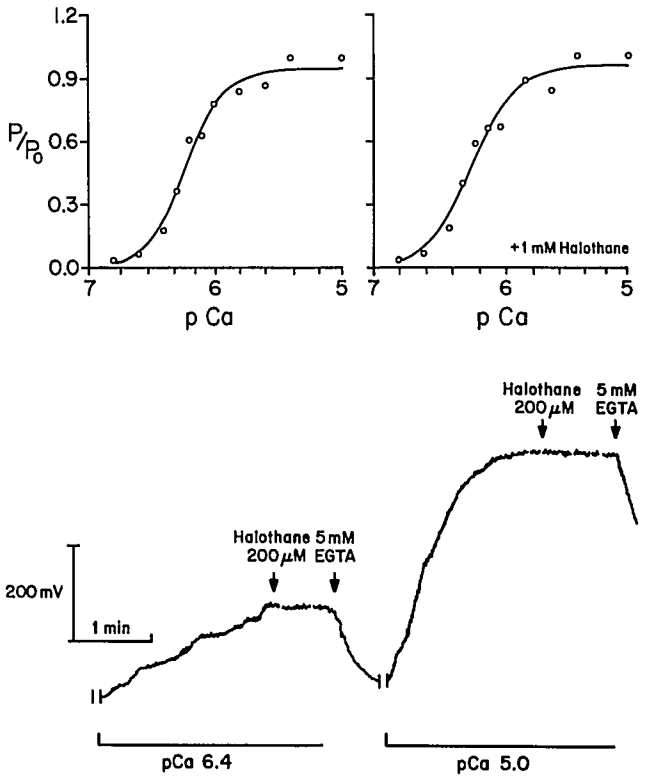

FIG. 5. *A*: The Ca^{2+}–tension curve of skinned cardiac bundle in the absence and presence of 1 mM (1.6%) halothane. The conditions and axes are the same as in figure 4. Each data point is the mean of three or four determinations. *B*: The effect of 200 μM (0.3%) halothane and 5 mM EGTA at *p*Ca of 6.4 and *p*Ca 5.0 on the tension of a skinned cardiac bundle.

that the overall conformation of cTnC with binding of Ca^{2+} to the high-affinity sites is not changed by halothane.

The important question, however, is whether the low-affinity, regulatory site is altered by halothane, resulting in a reduced response of cTNC to Ca^{2+}. Fuchs *et al.*[14] recently demonstrated that the binding of the sulfhydryl reagent, DTNB, to cysteine-35 and cysteine-84, which both reside in the N-terminal regulatory end of cTnC, is enhanced as Ca^{2+} binds to the low-affinity site. They demonstrated that the reaction rate of DTNB was increased twofold in response to saturation of the low-affinity regulatory site with Ca^{2+} and, therefore, could be used as a monitor of the Ca^{2+} responsiveness of the low-affinity site. We used this indirect approach to determine whether the low-affinity site was altered by halothane. Our experiments showed that halothane has no effect on the rate of DTNB binding; consequently, we can conclude that the N-terminal region of cTnC that contains the regulatory, low-affinity Ca^{2+}-binding site appears to be unaffected by halothane. Liou and Fuchs recently addressed one concern regarding the relevance of the reactivity of the sulfhydryl groups of isolated cTnC as a measure of the behavior of cTnC in the intact myofibril.[25] They showed that the binding of a sulfhydryl reagent to cTnC has a Ca^{2+} dependence in intact myofibrils similar to that in isolated cTnC.

The experiments with isolated cTnC, which were discussed above, demonstrated no effect of halothane on either the C-terminal, high-affinity binding sites or the N-terminal, low-affinity binding site. However, the possibility that the interaction of cTnC with the other troponin subunits or with the myofibrils might be modified by halothane still needed to be tested. We examined this possibility in two chemically skinned fiber systems, rabbit soleus and rabbit cardiac muscle.

The soleus is a slow skeletal muscle that contains the same isoform of TnC as cardiac muscle (*i.e.*, soleus TnC and cTnC are made by the same gene and have identical structures).[15] This system allowed us to ask the question whether cTnC in its native skeletal muscle environment with troponin I and troponin T, plus tropomyosin, myosin, and actin, demonstrated any modification in Ca^{2+} sensitivity or maximal contractile response when exposed to halothane. We found no change in Ca^{2+} sensitivity or maximal contractile response, further suggesting that the locus of negative inotropic effect by halothane was not at the TnC site. This is in agreement with Su's previous findings,[26] that muscle fibers containing the cardiac isoform of TnC showed no anesthetic sensitivity.

The rabbit skinned cardiac fiber was used to determine whether halothane affected the contractile apparatus at a site other than cTnC. Our data indicate no evidence of a negative inotropic effect of halothane either in terms of a change in Ca^{2+} sensitivity or the maximal Ca^{2+}-activated tension consistent with the experiments on isolated cTnC and soleus muscle. Our findings, however, are at variance with those of Murat *et al.*[7] and Su and Kerrick,[6] who observed very small but statistically significant effects of halothane. The experimental system of Su and Kerrick, who measured a 5% decrease in tension on mechanically disrupted fibers at a halothane concentration of 1% or higher, resembles more closely our experimental setup in that only the sarcolemmal membrane is rendered permeable. However, Murat *et al.* used fibers skinned with triton, which dissolves all membrane systems; they demonstrated a 2.5% change in *p*Ca and an 8.1% decrease in maximum tension at 1 MAC halothane.

At this time, we have no satisfactory explanation for the discrepancies between our findings and those of Murat *et al.* and Su and Kerrick. However, the rat skinned fiber used by Murat *et al.* exhibited considerably different characteristics than the rabbit fibers we examined. Murat *et al.*[7] obtained a value of 5.68 for the Ca^{2+} concentration at which 50% of maximal tension has been achieved

(pCa_{50}) for the rat cardiac fiber, whereas we obtained a pCa_{50} of 6.3 for the rabbit fiber. The 5-fold difference in Ca_{50} indicates a considerable difference in the Ca^{2+} sensitivity of the myofibrils of these two species and might relate to the difference in sensitivity to halothane. Furthermore, Su and Kerrick[6] reported a much lower pCa_{50} (5.25) for their rabbit skinned myocardial fiber, which is an order of magnitude lower than ours and indicates much lower sensitivity to Ca^{2+} than we have observed. These discrepancies suggest that it might be important to take a more intense look into species differences and experimental methods in understanding the action of volatile anesthetics on the myofibril.

In summary, we have found no evidence to support the hypothesis that cTnC or the cardiac myofibril is an important site of halothane's negative inotropic effect in rabbit ventricular myocardium.

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