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Comparison of the Effects of Halothane on Skinned Myocardial Fibers from Newborn and Adult Rabbit

I. Effects on Contractile Proteins

Elliot J. Krane, M.D., Judy Y. Su, Ph.D.†*

The effect of halothane on maximal and submaximal Ca^{2+}-activated tension development of the contractile proteins of newborn and adult cardiac muscle from rabbits was determined. Right ventricular muscle was removed from newborn and adult rabbits, and the sarcolemma was disrupted (skinned) by homogenization. Fiber bundles were dissected from the homogenate and mounted on tension transducers. Fiber bundles were alternately immersed in relaxing solution ($[Ca^{2+}] < 10^{-9}$ M) and contracting solutions (various $[Ca^{2+}]$ from $10^{-5.6}$ to $10^{-5.8}$ M), which were saturated with 100% N_2 alone or with three concentrations of halothane–N_2 mixture. In the absence of halothane, newborn skinned myocardial fibers were slightly more sensitive to submaximal Ca^{2+} concentrations than were adult myocardial fibers. $[Ca^{2+}]$ required for 50% maximum tension were $10^{-5.43}$ M and $10^{-5.51}$ M, respectively ($P < 0.05$). Halothane (1–3%) decreased the maximal Ca^{2+}-activated tension (at $[Ca^{2+}] = 10^{-5.8}$ M) similarly in adult and newborn myocardial fibers in a dose-dependent fashion. Tension was reduced by 5.9% for each 1% increase in halothane concentration. Halothane also decreased the sensitivity of adult myocardial skinned fibers to submaximal Ca^{2+} concentrations ($10^{-5.6}$ M to $10^{-5.0}$ M) by shifting the Ca^{2+}-tension response curve to the right. Only 3% halothane decreased the sensitivity of newborn myocardial skinned fibers to Ca^{2+}. The authors conclude that halothane causes less depression of Ca^{2+} activation of the contractile proteins in newborn than adult rabbit myocardium and that this effect of halothane cannot account for greater negative inotropy of halothane in the newborn. (Key words: Anesthesia: pediatric. Anesthetics, volatile: halothane. Heart: Contractile proteins; skinned fibers. Ions: calcium.)

HALOTHANE is a potent myocardial depressant at clinical concentrations in both infants[1–4] and adults.[5,6] Furthermore, many clinicians believe that halothane is a more potent hypotensive agent in newborn infants than in older children or adults.‡ Although direct clinical determination of age-related differences in sensitivity to halothane is not available, laboratory investigations have confirmed a greater depressant effect of halothane upon myocardial contractility in newborn rabbit,[7] kitten,[8] and rat[9] myocardium, compared with that from mature animals.

The cellular mechanism of the negative inotropic effect of halothane is not completely understood.[10] Because halothane is capable of diffusing into cells, it may affect cellular biochemistry at several loci by altering membrane function or by binding to intracellular protein sites and altering enzyme kinetics.[11] Although the effect of halothane upon the contractile protein complex actomyosin–ATPase may not be a major contributor to the overall mechanisms by which halothane depresses the adult myocardium,[10,12] age-dependent differences in the relative amounts of cardiac myosin isoenzymes and Ca^{2+}-dependent ATPase activity in newborn and adult myocardium[13] might in part account for the greater sensitivity of the newborn heart to halothane.

We hypothesized that the greater sensitivity of intact newborn myocardium[7–9] to halothane would result, in part, from greater sensitivity to halothane-induced depression of Ca^{2+} activation of the contractile proteins in the newborn. We tested this hypothesis by mechanically disrupting the sarcolemma of newborn and adult cardiac right ventricular muscle and controlling the halothane and Ca^{2+} concentrations surrounding the regulatory and contractile proteins. Accordingly, the effects of halothane on Ca^{2+}-activated tension development of the contractile proteins were investigated in these skinned fiber preparations.

Methods

Skinned Fiber Preparation

The experimental protocol was approved by the institution's Animal Experimentation Committee. The preparation of functionally skinned myocardial fiber preparations has been previously described in detail.[12,14] Newborn New Zealand white rabbits of either sex (age 1–4 days) were killed by decapitation. Young adult male New Zealand white rabbits (age 2 months, weight 1.5–2.5 kg) were killed by cervical dislocation. The hearts were rapidly isolated and cooled on ice. The right ventricle walls were dissected from the remainder of the heart and were cut into longitudinal strips. These strips were homogenized

* Assistant Professor, Departments of Anesthesiology and Pediatrics.
† Research Associate Professor, Department of Anesthesiology.

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Address reprint requests to Dr. Krane: Departments of Anesthesiology and Pediatrics, Children's Hospital and Medical Center, 4800 Sand Point Way, N.E., P.O. Box C-5371, Seattle, Washington 98105.

‡ Diaz JH, Lockhard CH: Is halothane really safe in infancy? (abstract). ANESTHESIOLOGY 51:S313, 1979.

in 5 ml relaxing solution (pCa = $-\log [Ca^{2+}]$ (M) > 9, see below) with a Teflon® pestle at the lightest pressure. The homogenate was placed in a glass-bottomed tray and diluted with approximately 5 ml of cold relaxing solution. Bundles of myocardial fibers approximately 100 μm in width, 20–30 μm thick, and 1–2 mm in length were isolated with the aid of a stereoscopic operating microscope and clamped with stainless steel clips. One end of the fiber bundle was attached to a photodiode tension transducer similar to that of Hellam and Podolsky[15] and the other end to a micromanipulator. The distance between the two clips was increased until slack in the fiber bundle was eliminated. Transducer signals were amplified and recorded on a Gould 2400S® four-channel recorder.

Bathing Solutions

The bathing solutions contained the following (in mM): Mg^{2+} = 1, K^+ = 35, Na^+ = 35, $MgATP^{2-}$ = 2, creatine phosphate^{2-} = 15, EGTA = 7, imidazole, and propionate (major anion). Ca^{2+} concentration varied in the solutions (pCa = 3.8, 5.0, 5.2, 5.4, 5.6, or >9). Ionic strength was kept constant at 0.15 and *p*H at 7.00 ± 0.02 by varying the amount of imidazole propionate at 23 ± 2° C. Total $[Ca^{2+}]$ in all solutions was measured by atomic absorption spectrophotometry (Hitachi 180-70®).

The high concentration of EGTA (7 mM) served to buffer small fluctuations in free intracellular $[Ca^{2+}]$, so that release or uptake of Ca^{2+} by intracellular organelles (for example, sarcoplasmic reticulum) would not alter the free intracellular $[Ca^{2+}]$. Thus, steady-state Ca^{2+}-activated tension development was controlled and was independent of sarcoplasmic reticulum function.

Partial pressures of halothane (Ayerst Laboratories) were controlled by directing reagent grade N_2 through a VerniTrol® vaporizer that had been previously calibrated by gas chromatography.[16] This halothane–N_2 mixture was then passed through a washing bottle containing distilled water for removal of particulate contaminants, and the gas was subsequently bubbled through the test bathing solutions. Earlier work from this laboratory demonstrated that halothane concentrations reached equilibrium in solution after 45 min of bubbling.[12]

Control bathing solutions were bubbled with 100% reagent grade N_2 alone, also after passing the gas through a separate washing bottle.

Gas flow through these solutions was stopped during immersion of the fiber bundle in the solution in order to avoid artifact. Previous work from this laboratory demonstrated that halothane loss to atmosphere from test solutions during tension measurements was approximately 0.02%/min,[12] which is insignificant compared with the range of halothane concentrations tested and the duration of tests.

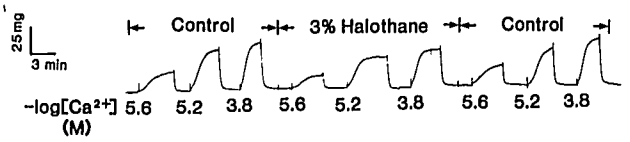

FIG. 1. Tracings showing the effect of 3% halothane on isometric tension development by newborn right ventricular skinned fibers in response to immersion in three Ca^{2+}-containing solutions (submaximal pCa = 5.6, 5.2 and maximal pCa = 3.8) alternating with relaxing solution (pCa > 9). Each experiment consists of immersing the preparation in two submaximal Ca^{2+} concentrations and one maximal Ca^{2+} concentration alternating with relaxing solution, followed by test solutions containing halothane, and finally in control solutions again.

Experimental Protocol

Adult and newborn fibers were studied simultaneously in pairs to assure identical experimental conditions. Two sequences were used for immersion of the fibers in Ca^{2+}-containing solutions. The first sequence alternated immersion in relaxing solution with immersion in two submaximal Ca^{2+} solutions (pCa = 5.6, 5.2) and maximal Ca^{2+} solution (pCa = 3.8) (fig. 1). The second sequence substituted the two submaximal Ca^{2+} solutions pCa = 5.4 and 5.0 and the same maximal Ca^{2+} concentration. Each myocardial fiber bundle was immersed in one sequence of control solutions (no halothane), then test solutions containing one of three concentrations of halothane (1%, 2%, or 3% v/v), then finally in control solutions again. This cycle was then repeated for the second sequence of solutions. Solution changes were made after steady-state tensions had been obtained.

Isometric tension development from baseline to steady state was compared between test solutions and the mean of the two bracketing control measurements, with the use of Student's *t* tests for paired data. The Ca^{2+} sensitivity (Ca^{2+} activation response curves) were determined from the equation:

$$\text{Tension}_i\ (\%\ \text{of maximum}) = \frac{(\text{absolute tension at pCa}_i)}{(\text{absolute tension at pCa} = 3.8)} \cdot 100\%$$

where i denotes corresponding points for halothane or control. These normalized data would eliminate differences between fibers in cross-sectional area. Each data point represented the mean of the results from six to nine skinned fibers, from at least three experimental animals.

Values for the pCa at which 50% maximal tension is obtained (the pCa_{50}) were interpolated with the use of linear regression analysis on the linear segment of the Ca^{2+} activation tension curve.

Data from newborn and adult cardiac preparations were compared with the use of Student's *t* tests for un-

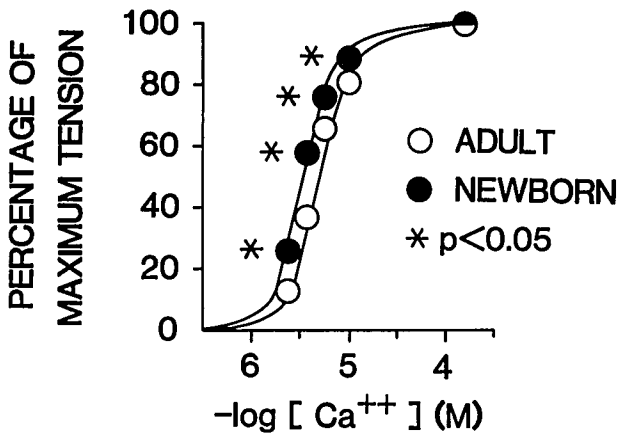

FIG. 2. Control Ca^{2+}-tension curves for adult (open circles) and newborn (closed circles) skinned cardiac fibers. Tension is expressed as a percentage of the maximum tension at pCa = 3.8. Each point represents the mean of six to nine skinned fiber preparations. *$P < 0.05$ compared with adult fibers.

paired data, after the arc-sine transformation was used to convert the data to a normal distribution.[17] One-way ANOVA followed by multiple t tests was used to determine significance of the dose response of maximal [Ca^{2+}] activation to halothane. $P < 0.05$ was regarded as statistically significant.

Results

Ca^{2+}-Tension Relationship

Comparison of absolute tension generation by adult and newborn cardiac skinned fibers was not determined because of our inability to accurately determine the cross-sectional area of the skinned fibers. However, there was no qualitative difference in tension development between newborn and adult.

The response of skinned fibers from the right ventricles of adult rabbits to Ca^{2+} (pCa = 5.6–3.8) showed increasing tension development with increasing [Ca^{2+}], resulting in a sigmoid Ca^{2+}-tension curve (fig. 2). The free [Ca^{2+}] required for 50% maximum tension (pCa_{50}) was 5.31, which is comparable to that observed in adult rabbit papillary muscle skinned fibers in a previous study.[12]

The Ca^{2+}-tension curve of the newborn was shifted toward lower Ca^{2+} concentrations (fig. 2). The pCa_{50} for newborn skinned fibers was 5.43 ($P < 0.05$ compared with adult). This indicates slightly greater sensitivity of the contractile proteins of the newborn to Ca^{2+}.

Effects of Halothane on the Ca^{2+}-Tension Relationship

Halothane depressed the contractile response to both submaximal and maximal [Ca^{2+}] (fig. 3), resulting in displacement of the Ca^{2+} tension curve. The resultant depression by halothane was statistically significant for each concentration of halothane tested (1%, 2%, and 3%) and for every Ca^{2+} concentration, except for pCa = 5.4 and 1% halothane in the newborn and pCa = 5.0 and 1% halothane in the adult. The downward and rightward displacement of the tension curves by halothane indicated both loss of sensitivity of the contractile proteins to Ca^{2+} (resulting in rightward displacement, see below) and inhibition of the strength of contraction by maximal Ca^{2+} concentration (resulting in downward displacement).

The depression of maximal Ca^{2+}-activated tension (at pCa = 3.8) by halothane was dose dependent and similar in newborn and adult (fig. 4). Analysis of variance showed that from 1 to 2%, halothane caused significant depression of tension, whereas there was a trend toward further depression from 2 to 3% halothane, although the difference was not statistically significant. Linear regression analysis revealed that each 1% increment of halothane concentration decreased developed tension at pCa = 3.8 by 5.9%, which is comparable to the response of adult rabbit skinned papillary muscle fibers observed in a pre-

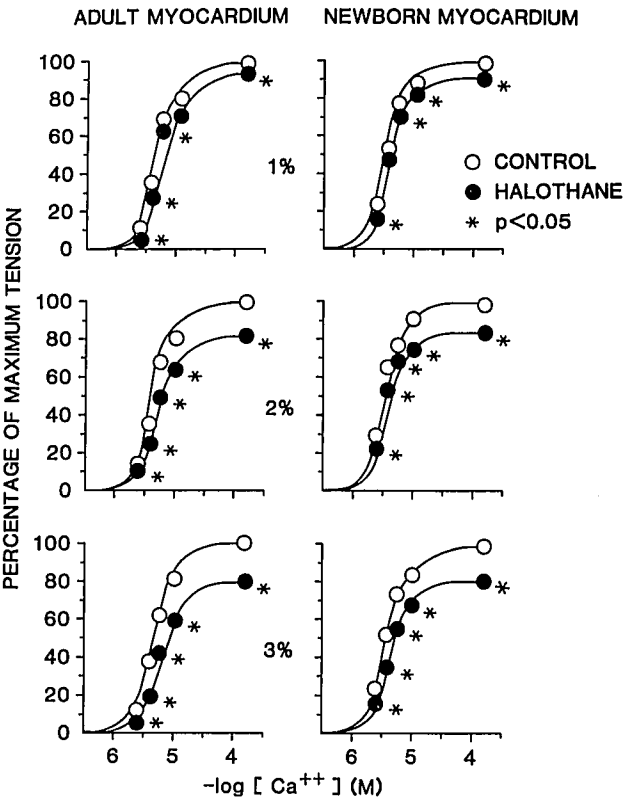

FIG. 3. The effect of halothane (1–3%) on the Ca^{2+}-tension curves of adult skinned right ventricular fibers (left panel) and newborn skinned right ventricular fibers (right panel). Open circles: control (no halothane). Closed circles: halothane. *$P < 0.05$ compared with control.

vious study.[13] There was no statistical difference between the responses of newborn and adult skinned fibers to maximal [Ca^{2+}] in the presence of halothane.

Normalizing the data in figure 3 to maximal tension at each concentration of halothane yielded new Ca^{2+}–tension curves for each concentration of halothane tested (fig. 5) and allows analysis of the sensitivity of the preparations to Ca^{2+} in the presence of halothane. A decrease in Ca^{2+} sensitivity is thus seen as a shift of the tension curve to the right. Halothane (1–3%) decreased the sensitivity of adult skinned myocardium to Ca^{2+}, such that with increasing halothane concentrations, higher Ca^{2+} concentrations were required for 50% activation. The effect of halothane upon the Ca^{2+}–tension curve was less prominent in newborn skinned fibers; only 3% halothane increased the Ca^{2+} concentration required for 50% activation.

The effects of halothane upon Ca^{2+}-activated tension development of the contractile proteins were reversible (fig. 1).

Discussion

The Ca^{2+} activation of the contractile proteins in skinned fibers from newborn rabbits was not more sensitive to halothane than in skinned fibers from adult rabbits. Two discrete effects of halothane on skinned fibers were observed. Halothane (1–3%) directly depressed maximal Ca^{2+}-activated tension development in newborn and adult myocardial skinned fibers equally in a dose-dependent manner, resulting in a downward displacement of the Ca^{2+}–tension curve. Halothane also depressed submaximal Ca^{2+}-activated tension development in adult skinned fibers, resulting in a shift of the normalized Ca^{2+}–tension curves toward higher Ca^{2+} concentrations. However, this latter effect was less in skinned fibers from newborn rabbits than from adults and was demonstrable only with 3% halothane. These observations refute our initial hypothesis and demonstrate that the newborn contractile proteins are not the locus of halothane's greater cardiac depressant effect in the newborn.

New Zealand white rabbits were selected for this study because of our laboratory's extensive experience with the skinned fiber model isolated from this species and because comparisons of the cardiovascular response of the newborn New Zealand white rabbit show both a greater hypotensive effect of halothane in the newborn compared with the adult rabbit and loss of the baroreceptor response in the newborn rabbit,[18] mirroring clinical observations in the newborn human.[4,19]

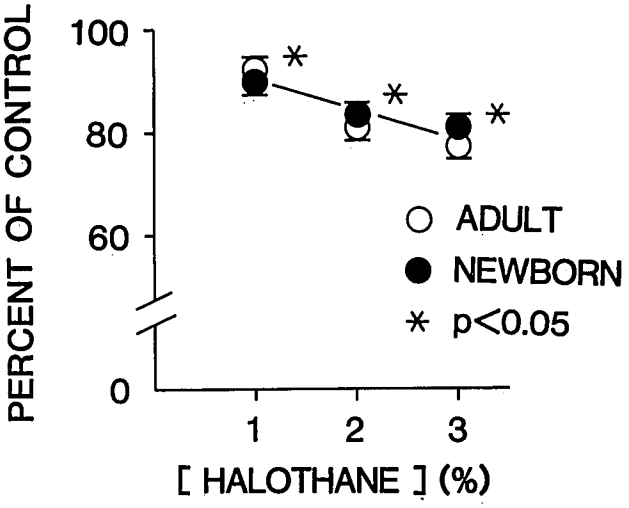

FIG. 4. Effect of halothane (mean ± SEM) on newborn (closed circles) and adult (open circles) right ventricular skinned fiber tension development at pCa = 3.8 (*e.g.*, maximal [Ca^{2+}]). Tension is expressed as a percentage of the control tension development at pCa = 3.8 in the absence of halothane. *$P < 0.05$ compared with control. There is no statistical difference between newborn and adult fibers nor between 2% and 3% halothane.

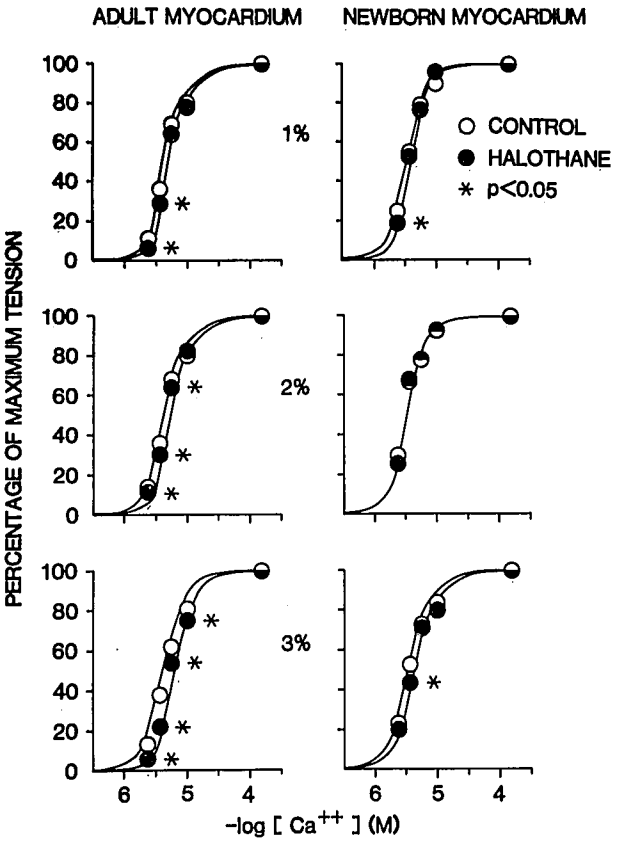

FIG. 5. Effects of halothane (1–3%) on the Ca^{2+}–tension curve of adult skinned right ventricular fibers (left panel) and newborn skinned right ventricular fibers (right panel). Curves are normalized to 100% as maximum tension for both control (open circles) and halothane (closed circles). *$P > 0.05$ compared with control.

In the absence of halothane, newborn skinned myocardial fibers had slightly greater sensitivity to free Ca^{2+} than did adult skinned fibers. The pCa associated with 50% of maximal tension generation was 0.12 units higher in the newborn rabbit than in the adult. This finding is in agreement with earlier data of Fabiato,[20] which showed a shift of the newborn rat ventricle Ca^{2+}–tension curve about 0.15 pCa units to the left of the adult rat ventricle curve. The explanation for this phenomenon is presently unknown but may relate to differences in the affinity of troponin-C to Ca^{2+} in newborn and adult myocardium. The greater sensitivity of the newborn to intracellular Ca^{2+} activation may serve to facilitate direct activation of newborn myofibrils by Ca^{2+} derived from transsarcolemmal Ca^{2+} influx, which is consistent with current hypotheses that the newborn myocardium derives a greater proportion of activator Ca^{2+} from the extracellular pool rather than from the intracellular pool (*e.g.*, sarcoplasmic reticulum), as is the case for adult mammalian myocardium.[21,22]

In the presence of halothane, depression of maximal Ca^{2+}-activated tension development can be interpreted as an effect of halothane on the number of, or strength of, cross-bridge interactions. In our preparation, 3% halothane depressed maximal tension development (that is, at pCa = 3.8) by approximately 20%. Consistent with the findings of Merin *et al.*,[23] who found 20% reversible depression of dog cardiac actomyosin ATPase that was exposed to 2 mM halothane (equivalent to 4.5% halothane in the gas phase), we demonstrated similarly decreased maximal tension development, which would be expected to be associated with depression of actomyosin ATPase activity.

Myofibrillar ATPase activity is affected by developmental changes, and it differs in atrial *versus* ventricular myocardium; this probably reflects the different types of myosin isoenzyme present in different cardiac chambers or at different stages of development.[13,24] Myocardium isolated from fetal or newborn hearts contains two forms of myosin isoenzyme: one form, V1, with high Ca^{2+}-dependent ATPase activity; and another, V3, associated with lower Ca^{2+}-activated and actin-activated ATPase activities.[25,26] Normal adult mammalian myocardium is predominantly composed of the V1 form of myosin heavy chain only and therefore has faster ATPase activity than newborn myocardium.

Despite this difference in myosin and myofibrillar ATPase activity, newborn and adult rabbit skinned fibers demonstrated identical halothane-induced depression of maximal Ca^{2+}-activated tension. Therefore, we conclude that, although myosin isoenzymes may play a partial role in determining the overall contractile properties of the newborn and adult heart, in all probability they do not explain developmental differences found in sensitivity to halothane.

Ohnishi *et al.*[27] demonstrated reversible depression of cat myofibrillar ATPase by 1 and 2% halothane. They suggested that the locus of activity of halothane on the contractile proteins was at troponin–tropomyosin binding of Ca^{2+} because chemical removal of the Ca^{2+} regulatory protein complex troponin–tropomyosin (by washing with 2 mM $NaHCO_3$) rendered the cardiac myofibrillar ATPase insensitive to the depressant effect of halothane. If troponin–tropomyosin binding of intracellular Ca^{2+} is indeed one site at which halothane depresses the contractile proteins, our results that show less effect of halothane on newborn Ca^{2+} sensitivity suggest that maturational changes in troponin make adult cardiac contractile proteins more, rather than less, sensitive to the effects of halothane on Ca^{2+} binding by troponin.

When intact (unskinned) right ventricular muscle strips are exposed to about 1% halothane, maximal isometric tension generation and rate of tension development are both depressed by approximately 60% in adult rabbit myocardium and 70% in newborn rabbit myocardium.[7] Similar results are seen in rat and cat myocardium.[8,9] In contrast, exposure of newborn and adult rabbit myocardial skinned fibers to 1% halothane in the present study resulted in only 10% depression of maximal Ca^{2+}-activated tension (fig. 4). Therefore, in both newborn and adult myocardium, direct depression of Ca^{2+}-activated tension development can account for only a relatively small proportion of total halothane-induced depression of tension development. Although the act of disrupting the myocardial sarcolemma by mechanical skinning may result in some loss or alteration of the myofibrillar regulatory mechanisms (specifically troponin) and its response to anesthetics, attenuating measureable differences in newborn and adult skinned fiber response to Ca^{2+} in the presence of anesthetics, it is unlikely that significant loss of troponin occurred from our preparation. Troponin extraction requires detergent skinning of the sarcolemma, and extraction in relatively alkaline solutions of low ionic strength over a long period of time,[28] conditions very different than those used to skin myocardial fibers in the present study.

Other mechanisms for halothane-induced negative inotropy have been identified in adult myocardial preparations, particularly depression of uptake and release of Ca^{2+} by the sarcoplasmic reticulum[14,29] and alteration of sarcolemmal Ca^{2+} exchange.[30] Finally, Krane and Su have shown that newborn sarcoplasmic reticulum is not more sensitive to halothane effects than is adult,[31] but no information is presently available regarding the effect of anesthetics on physiologic mechanisms of Ca^{2+} release from the sarcoplasmic reticulum nor regarding the behavior of the newborn myocardial sarcolemma when exposed to anesthetic agents.

In summary, newborn and adult rabbit right ventricular skinned fibers activated by maximal [Ca^{2+}] were seen to

have similar depression when exposed to 1–3% halothane. Newborn skinned fibers activated by submaximal [Ca^{2+}] exhibited less halothane-induced depression than did adult skinned fibers. Our observations indicate that developmentally immature cardiac contractile proteins, myofibrillar ATPase, and their regulatory proteins cannot account for the larger negative inotropic effect of halothane seen in intact newborn myocardial preparations, young animals, and human infants. This suggests that such an effect results from a greater action of halothane on an as yet undefined cellular locus.

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