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Mechanisms Underlying Greater Sensitivity of Neonatal Cardiac Muscle to Volatile Anesthetics

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Background: **In neonatal heart, plasma membrane Na^+–Ca^{2+} exchange (NCX) and Ca^{2+} influx channels play greater roles in intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ regulation compared with the sarcoplasmic reticulum (SR). In neonatal (aged 0–3 days) and adult (aged 84 days) rat cardiac myocytes, we determined the mechanisms underlying greater sensitivity of the neonatal myocardium to inhibition by volatile anesthetics.**

Methods: **The effects of 1 and 2 minimum alveolar concentration halothane and sevoflurane on Ca^{2+} influx during electrical stimulation in the presence or blockade of NCX and the Ca^{2+} channel agonist BayK8644 were examined. $[Ca^{2+}]_i$ responses to caffeine were used to examine anesthetic effects on SR Ca^{2+} release (*via* ryanodine receptor channels) and reuptake (*via* SR Ca^{2+} adenosine triphosphatase). Ca^{2+} influx *via* NCX was examined during rapid activation in the presence of the reversible SR Ca^{2+} adenosine triphosphatase inhibitor cyclopiazonic acid and ryanodine to inhibit the SR. Efflux mode NCX was examined during activation by extracellular Na^+ in the absence of SR reuptake.**

Results: **Intracellular Ca^{2+} concentration transients during electrical stimulation were inhibited to a greater extent in neonates by halothane (80%) and sevoflurane (50%). Potentiation of $[Ca^{2+}]_i$ responses by BayK8644 (160 and 120% control in neonates and adults, respectively) was also blunted by anesthetics to a greater extent in neonates. $[Ca^{2+}]_i$ responses to caffeine in neonates (~30% adult responses) were inhibited to a lesser extent compared with adults (35 *vs.* 60% by halothane). Both anesthetics inhibited Ca^{2+} reuptake at 2 minimum alveolar concentration, again to a greater extent in adults. Reduction in NCX-mediated influx was more pronounced in neonates (90%) compared with adults (65%) but was comparable between anesthetics. Both anesthetics also reduced NCX-mediated efflux to a greater extent in neonates. Potentiation of NCX-mediated Ca^{2+} efflux by extracellular Na^+ and NCX-mediated Ca^{2+} influx by intracellular Na^+ were both prevented by halothane, especially in neonates.**

Conclusions: **These data indicate that greater myocardial depression in neonates induced by volatile anesthetics may be mediated by inhibition of NCX and Ca^{2+} influx channels rather than inhibition of SR Ca^{2+} release.**

COMPARED with the adult muscle, neonatal cardiac muscle has been shown to be more sensitive to depression by volatile anesthetics.[1–3] Relatively greater anesthetic effects on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in neonatal cardiac myocytes may underlie greater myocardial depression. The precise targets of anesthetic action, especially in the neonate, are still being evaluated.

In the adult heart, in addition to sarcoplasmic reticulum (SR) release, which has the greatest contribution to activating Ca^{2+} for contraction, Ca^{2+} fluxes across the plasma membrane are necessary for activation of such SR Ca^{2+} release during systole. Influx of Ca^{2+} occurs through influx channels such as L-type channels, as well as the plasmalemmal Na^+–Ca^{2+} exchanger (NCX). Relaxation is mediated through replenishment of SR Ca^{2+} *via* the sarcoendoplasmic reticulum Ca^{2+} adenosinde triphosphatase (SERCA), as well as Ca^{2+} extrusion across the plasma membrane. In this regard, the plasmalemmal NCX is thought to be a key player during cardiac muscle relaxation, facilitating Ca^{2+} efflux while operating in a "forward" mode where extracellular Na^+ is brought into the myocyte in exchange for Ca^{2+} (see Blaustein and Lederer[4] for review). Some studies have suggested that, in conjunction with Ca^{2+} influx channels, NCX also plays a role in $[Ca^{2+}]_i$ elevation (operating in "reverse" mode by extruding intracellular Na^+).[5,6]

In contrast to the adult heart, the neonatal heart is thought to be generally more dependent on Ca^{2+} fluxes across the plasma membrane through L- and T-type Ca^{2+} channels,[7,8] with the contribution of intracellular SR Ca^{2+} stores to total $[Ca^{2+}]_i$ being smaller. Some studies suggest that Ca^{2+} influx channels alone are insufficient for providing Ca^{2+} for muscle contraction in neonatal heart.[9–11] Accordingly, it has been suggested that NCX-mediated Ca^{2+} influx plays a physiologic role in the neonatal heart.[10] Furthermore, given an immature SR, NCX-mediated Ca^{2+} efflux may be key to relaxation of the neonatal heart.

Given a greater dependence of the neonatal heart on Ca^{2+} fluxes across the plasma membrane, we hypothesized that effects on Ca^{2+} influx channels and on NCX, rather than effects on the SR, underlie greater volatile anesthetic sensitivity of neonatal myocardium. Studies in the adult heart have demonstrated that inhibition of Ca^{2+} influx channels,[12–14] as well as SR Ca^{2+} release *via* ryanodine receptor (RyR) channels,[15–17] contribute to anesthetic-induced myocardial depression. In a recent study in the adult rat heart, we demonstrated that halothane, and to a lesser extent sevoflurane, inhibited NCX-mediated Ca^{2+} influx.[18] However, similar studies in the neonate are lacking. On the other hand, inhibition of Ca^{2+} fluxes across the plasma membrane by anesthetics may increase the relative contribution of SR Ca^{2+} to total $[Ca^{2+}]_i$. Therefore, it is also necessary to compare anesthetic effects on SR Ca^{2+} release and reuptake. Accord-

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ingly, in the current study we used enyzmatically dissociated single ventricular myocytes from neonate (aged 0–3 days) *versus* adult (aged ≥ 84 days) rats to compare the effects of clinically relevant concentrations of halothane *versus* sevoflurane on Ca^{2+} influx channels, NCX, as well as SR Ca^{2+} release and reuptake.

Methods

Animals

All procedures involving animal use were approved by the Institutional Animal Care and Use Committee of the Mayo Clinic (Rochester, MN). Hearts from 3–4 Sprague-Dawley pups (neonates, < 3 days postpartum; mean body weight, 7 ± 2 g) were combined for one experiment (total 50 dissociations). A total of 42 male rats (at least 12 weeks of age; 250–300 g body weight) formed the adult group.

Animals were anesthetized with intramuscular ketamine (60 mg/kg) and xylazine (2.5 mg/kg). The hearts were excised and rapidly immersed in oxygenated (95% O_2, 5% CO_2) ice-cold Krebs solution (118.3 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.0 mM $CaCl_2$, 11 mM glucose; pH 7.40).

Dissociation of Cardiac Myocytes

Adult cardiac myocytes were enzymatically dissociated using a Langendorf perfusion-based technique.[18] The heart was sequentially perfused with Dulbecco minimum essential medium (Joklik modification with 10 mM KCl and 10 mM HEPES; Sigma Chemicals, St. Louis, MO) containing 1 mM Ca^{2+}, 0 Ca^{2+}, and 80 mg/ml Type I collagenase (Worthington Chemicals, Lakewood, NJ) with 1% bovine serum albumin (Sigma) in 0 Ca^{2+}. During collagenase perfusion, Ca^{2+} was added back in graded steps to 1 mM. The tissue was gently triturated to obtain single myocytes, which were finally washed in Joklik medium with 1% albumin and 1 mM Ca^{2+}.

In pilot studies on 3–4 animals, we found that aortic cannulation in neonates was technically difficult and unreliable. In further studies on 3–4 animals, we used a commercially available neonatal myocyte isolation kit (Worthington), compared resting $[Ca^{2+}]_i$ and responses to 5 mM caffeine in cells isolated using the two techniques, and found the responses to be less than 10% different. Accordingly, we used the isolation kit for dissociation of neonatal myocytes for the remainder of the study. Ventricles from 3–4 animals were placed directly in ice-cold Ca^{2+} and Mg^{2+} free Hanks balanced salt solution (Sigma), minced with scissors, washed, and incubated overnight at 4°C with 50 μg/ml trypsin. A soybean trypsin inhibitor was then added, and the tissue was oxygenated for 1 min. Subsequently, the samples were warmed to 37°C and incubated further for 1 h with 50 mg/ml collagenase. The cells were finally washed and processed in the same manner as adult myocytes.

Myocytes were plated on laminin-coated glass coverslips and kept at 37°C for a maximum of 5 h, within which time daily experiments were completed. Myocyte viability and Ca^{2+} tolerance were evaluated as described previously.[18] Before data analyses, the following exclusion criteria for cells were applied: (1) greater than 50 nM variation in resting $[Ca^{2+}]_i$ at the initiation of a protocol; (2) greater than five spontaneous $[Ca^{2+}]_i$ transients per minute (noted in ~15% of cells); (3) greater than 10% change in resting $[Ca^{2+}]_i$ during intervening washes; and (4) greater than 10% dye bleaching over a 5-min period of laser exposure.

Confocal Intracellular Ca^{2+} Concentration Imaging

We previously published detailed descriptions of the real-time confocal imaging techniques for cardiac myocytes.[18] Myocytes incubated in 5 μM fluo-3/AM (Molecular Probes, Eugene, OR) were imaged using a Noran Odyssey XL real-time confocal system (Noran Instruments, Middleton, WI) on a Nikon Diaphot inverted microscope (Nikon Instruments USA, New York, NY). Normal Tyrode medium containing 145 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES (pH 7.4; 25°C) was used initially. A fiberoptic fluid-level controller (ALA Instruments, Westbury, NY) was used to minimize the influence of the fluid level on NCX activation, threshold for electrical stimulation, and agonist concentration. Images (640 × 480 pixels) were acquired at 30 frames/s at 400× magnification (Olympus 40X/1.3 oil-immersion lens; Olympus USA, Los Angeles, CA) and an optical section thickness of 1 μm. The $[Ca^{2+}]_i$ responses were obtained using individual software-defined regions of interest for two to three cells.

Empirical calibration of fluo-3 fluorescence levels for $[Ca^{2+}]_i$ was performed using previously described techniques,[18] based on sequential exposure to known Ca^{2+} concentrations (0 nM to 1.25 μM; Molecular Probes Calcium Calibration Buffer Kit; Molecular Probes, Eugene, OR), and 10 μM A-23187 (Ca^{2+} ionophore) to allow equilibration of $[Ca^{2+}]_i$ and extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$). An empirical calibration curve was then generated and compared with measurements by other investigators using the following equation:

$$[Ca^{2+}]_i = K_d \frac{F - F_{min}}{F_{max} - F}$$

to calculate $[Ca^{2+}]_i$ from fluorescence values (F), where F_{min} is the fluorescence at minimal $[Ca^{2+}]_i$ (0 nM in this study) and F_{max} at saturating concentrations, determined using a buffer and ionophore technique as above.[19] Using the F_{min}, F_{max} (expressed as gray levels ranging from 0 to 254), we estimated the apparent K_d for fluo-3 to be 522 ± 101 nM in adults and 504 ± 89 nM in neonates. Although these values are somewhat higher than the 400 nM used in previous studies,[19] it must also be recognized that the K_d for fluo-3 can differ *in vivo versus in vitro*.[20]

Volatile Anesthetics

As in previous studies,[18] a calibrated online vaporizer was used to add halothane (Wyeth-Ayerst Laboratories, Philadelphia, PA) and sevoflurane (Abbott Laboratories, Deerfield, IL) to the aerating gas mixture. Aqueous anesthetic concentrations equivalent to 1 and 2 adult rat minimum alveolar concentration (MAC) at room temperature (25°C) were measured for halothane and were determined by gas chromatography and an electron capture detector (Hewlett-Packard 5880A; Hewlett-Packard, Sunnyvale, CA)[21] and for sevoflurane using a flame ionization detector. Halothane concentrations were 0.32 ± 0.11 mM for 1 MAC and 0.50 ± 0.11 mM for 2 MAC, and sevoflurane concentrations were 0.48 ± 0.10 mM and 0.70 ± 0.11 mM for 1 and 2 MAC, respectively.

Effect of Volatile Anesthetics on Ca^{2+} Influx

Anesthetic Effects on Intracellular Ca^{2+} Concentration Responses to Electrical Stimulation. Myocytes perfused with Joklik medium containing 1 mM Ca^{2+} were electrically stimulated at 0.25 Hz, 5 ms using two platinum wires within the microscope chamber. Stimulation was maintained for 2 min to ensure $[Ca^{2+}]_i$ stability. However, to minimize dye bleaching, recordings were made only every 30 s for two to three stimulations. Cells were subsequently perfused with Joklik medium containing 1 or 2 MAC volatile anesthetic, and the $[Ca^{2+}]_i$ responses were recorded for 3 min, after which recovery from anesthetic was evaluated for 1 min. In control experiments, cells were electrically stimulated for 5 min with no anesthetic exposure.

Volatile Anesthetic Effects on Intracellular Ca^{2+} Concentration Responses to BayK8644. Electrically stimulated myocytes were exposed for 3 min to 1 μM BayK8644 (Sigma), a selective activator of L-type Ca^{2+} influx channels. In the presence of BayK8644, cells were perfused with anesthetics for an additional 2 min, followed by washout for 1 min. Control cells were only exposed to BayK8644 for 5 min.

In a second set of experiments, myocytes were preexposed to 10 μM ryanodine (Sigma), 5 μM thapsigargin (Sigma), and 10 μM KBR 7943 (Tocris Corp., Ellisville, MO). The combination of ryanodine and thapsigargin ensured inhibition of SR Ca^{2+} release as well as reuptake, thus functionally isolating the plasma membrane. KBR 7943 is a novel drug that has been reported to inhibit NCX.[22] During these conditions, the contribution of Ca^{2+} influx channels to the $[Ca^{2+}]_i$ response to electrical stimulation could be determined. Myocytes were electrically stimulated for an additional 2 min, exposed to anesthetics for 2 min, and washed for 1 min. Control cells were stimulated for 5 min.

In a third set of experiments, myocytes preexposed to ryanodine, thapsigargin, and KBR 7943 were exposed to 1 μM BayK8644 for 2 min. Lastly, cells were perfused with anesthetics for an additional 2 min, and their response to electrical stimulation was recorded.

Effect of Volatile Anesthetics on Sarcoplasmic Reticulum Ca^{2+} Release

Volatile Anesthetic Effects on Intracellular Ca^{2+} Concentration Responses to Caffeine. Myocytes were exposed to 5 mM caffeine in the presence of 2 mM extracellular Ca^{2+}, and $[Ca^{2+}]_i$ responses were recorded. Cells were washed for 10 min to replenish SR Ca^{2+} stores and then reexposed to caffeine in the presence of anesthetic. The length of time for SR refilling was kept constant across all experiments.

In a second set of experiments, myocytes were preexposed to zero extracellular Ca^{2+}, low (4 mM) Na^+, 1 mM lanthanum chloride (La^{3+}), and 10 μM KBR 7943. This combination functionally isolated the SR by blocking Ca^{2+} influx channels, NCX, and Ca^{2+} efflux *via* the plasma membrane Ca^{2+} adenosine triphosphatase (PMCA; inhibited by La^{3+}). Cells were preexposed for 5 min to 10 μM cyclopiazonic acid (CPA; Sigma), a selective and reversible inhibitor of SERCA. Subsequently, SR Ca^{2+} release was induced by 5 mM caffeine. The rise time of the $[Ca^{2+}]_i$ response (normalized for amplitude) was taken to represent the rate of SR Ca^{2+} release, and the amplitude as an index of SR Ca^{2+} content. Cells were washed for 15 min to allow SR replenishment and were then reexposed to CPA in the absence (control) or presence of volatile anesthetics for 5 min, followed by caffeine in the continued presence of CPA and anesthetic.

Effect of Volatile Anesthetics on Sarcoplasmic Reticulum Ca^{2+} Reuptake

Myocytes preexposed to zero extracellular Ca^{2+}, low Na^+, La^{3+}, and KBR 7943 were exposed to 5 mM caffeine. The fall time of the $[Ca^{2+}]_i$ response was taken to represent the rate of Ca^{2+} reuptake *via* SERCA. The contribution of Ca^{2+} efflux *via* PMCA and NCX was assumed to be minimal in the presence of La^{3+} and low extracellular Na^+. The cells were then washed for 15 min, and the protocol was repeated in the absence (control) or presence of volatile anesthetics.

Estimation of Sarcoplasmic Reticulum Volume Density

In a separate set of animals, cardiac ventricles were carefully separated, washed, stretched and pinned on cork, and rapidly frozen in melting isopentane cooled by liquid nitrogen. Cross-sections (10 μm) cut using a cryostat (Reichert-Jung Model 2000E, Germany; −20°C) were placed on slides and incubated for 10 min in 1 μM Bodipy-tetramethylrhodamine conjugated ryanodine (Molecular Probes) in 0.1 M phosphate buffer to stain the SR. Sections were then fixed in 2% paraformaldehyde in phosphate buffer, washed, and coverslipped. Sections for staining control were directly fixed without expo-

sure to ryanodine. The samples were visualized at 400× using a BioRad MRC500 laser confocal microscope (Bio-Rad Instruments, Hercules, CA) equipped with an Ar-Kr laser (568-nm line for excitation of rhodamine, 590-nm emission filter), at a resolution of 0.3 μm/pixel. Areas of fluorescence above a background threshold (determined from control samples) were considered to be areas of SR. This measured area was expressed as a fraction of myocyte area. At least 20 myocytes were sampled from each animal.

Effects of Volatile Anesthetics on Na^+-Ca^{2+} Exchange in Neonates versus *Adults*

Influx Mode Na^+–Ca^{2+} Exchange. The technique for evaluating the effect of volatile anesthetics on influx mode of NCX was recently published[18] and is also illustrated in figure 1. Cells with stable, resting $[Ca^{2+}]_i$ were "Na-loaded" with Tyrode solution containing 0 Ca^{2+} (5 mM EGTA) and normal Na^+, 5 μM CPA and 10 μM ryanodine (Sigma), a specific RyR channel inhibitor, thus functionally isolating the plasma membrane. Because the major mechanisms of Ca^{2+} influx across the plasma membrane were blocked by 0 Ca^{2+}, namely, Ca^{2+} influx channels and NCX, $[Ca^{2+}]_i$ was expected to remain stable. After Na^+ loading cells for 1 min, perfusion was rapidly switched (< 300 ms) to Tyrode solution containing 0 Na^+, normal Ca^{2+}, CPA, and ryanodine, selectively activating the influx mode of NCX, reflected by the rate of increase of $[Ca^{2+}]_i$. After 1 min, both CPA and ryanodine were washed out with normal Tyrode solution. No attempt was made to determine whether ryanodine was actually washed out because it was not expected to adversely affect subsequent manipulations. The protocol was then repeated in the absence of (control) or with preexposure to volatile anesthetic before rapid NCX activation.

Efflux Mode Na^+–Ca^{2+} Exchange. To examine the effect of volatile anesthetics on the efflux mode of NCX (fig. 1), cells initially perfused with normal Tyrode solution were switched to solution containing 0 Na^+, 0 Ca^{2+} (with 5 mM EGTA), and 5 μM CPA. Thus, the major mechanisms for decreasing $[Ca^{2+}]_i$ (SERCA and NCX) as well as Ca^{2+} influx were inhibited. Accordingly, $[Ca^{2+}]_i$ was expected to increase because of continued SR Ca^{2+} "leak." A potential mechanism for Ca^{2+} extrusion not inhibited was the PMCA. However, in our experience the contribution of PMCA to $[Ca^{2+}]_i$ during this protocol is fairly insignificant and can be ignored (see Discussion). After $[Ca^{2+}]_i$ was allowed to increase for approximately 1–2 min (and more or less stabilized), efflux mode NCX was rapidly (< 300 ms) and selectively activated by Tyrode solution containing 0 Ca^{2+} (with EGTA), normal Na^+, and CPA. The rapid rate of decrease in $[Ca^{2+}]_i$ was recorded as an index of NCX efflux rate. When $[Ca^{2+}]_i$ reached baseline, the CPA was washed out for 5 min, allowing SR replenishment. The protocol was then repeated in the presence or absence of 1 or 2 MAC volatile anesthetic in the 0 Na^+–0 Ca^{2+} and 0 Ca^{2+}–normal Na^+ solutions.

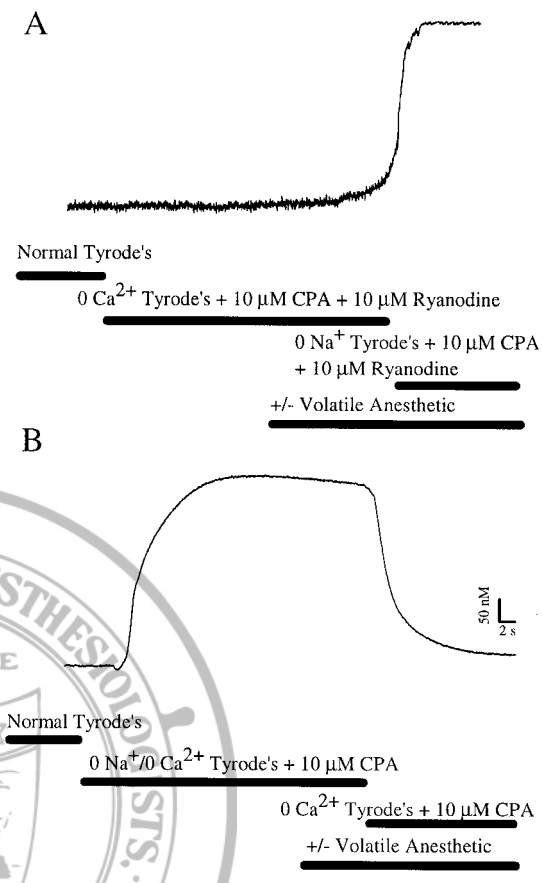

Fig. 1. (*A*) Effect of volatile anesthetics on influx mode Na^+–Ca^{2+} exchange (NCX) in single rat cardiac myocytes loaded with the Ca^{2+} indicator fluo-3. Cells were "Na^+-loaded" during conditions of 0 Ca^{2+} and blocked sarcoplasmic reticulum (SR; ryanodine and cyclopiazonic acid [CPA]). Influx mode NCX was selectively activated by rapid reintroduction of extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) and simultaneous removal of Na^+ in the presence or absence of volatile anesthetic. The rate of increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured as an index of NCX activity. (*B*) Effect of volatile anesthetics on efflux mode NCX. Cells were exposed to 0 Na^+–0 Ca^{2+} to inhibit efflux mode NCX and CPA to inhibit Ca^{2+} reuptake. During these conditions, $[Ca^{2+}]_i$ was allowed to increase. Efflux mode NCX was then selectively activated by rapid introduction of extracellular Na^+ concentration ($[Na^+]_o$) in the presence or absence of volatile anesthetic. The rate of decrease in $[Ca^{2+}]_i$ was measured as an index of NCX-mediated efflux.

Na^+ Dependence of Na^+–Ca^{2+} Exchange. The effect of volatile anesthetics on the relation between Na^+ gradient and influx mode of NCX was examined by Na^+-loading cells with Tyrode solution containing 0 Ca^{2+}, CPA, ryanodine, and one of three Na^+ concentrations (35, 70, or 145 mM). The same cell was used for all three concentrations. Cells were exposed to volatile anesthetics as in the previous protocol. The effect of volatile anesthetics on the relation between Na^+ concentration and efflux mode of NCX was examined by introducing Tyrode solution containing 0 Ca^{2+} and one of

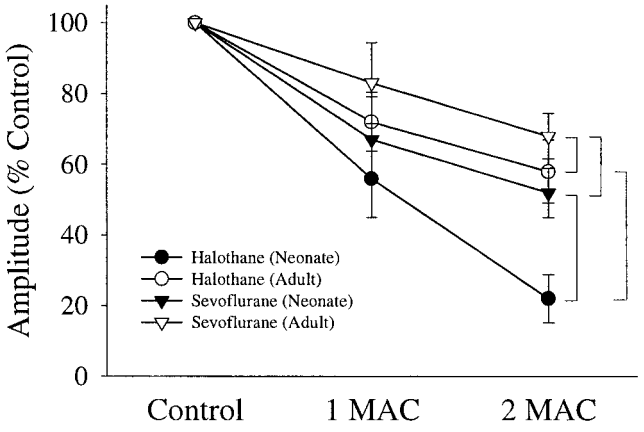

Fig. 2. Effect of volatile anesthetics on the intracellular Ca^{2+} concentration response to electrical stimulation (0.25 Hz, 5 ms) in neonatal *versus* adult cardiac myocytes. Myocytes were exposed to 1 or 2 minimum alveolar concentration (MAC) of halothane or sevoflurane. Brackets indicate statistically significant difference in pairwise comparisons ($P < 0.05$). Values are mean ± SD.

three different concentrations of Na^+ (35, 70, or 145 mM) during rapid activation of NCX in the presence or absence of anesthetic. Cell viability was tested at the end of the protocol by repeating the protocol with 145 mM Na^+.

Statistical Analysis

At least 15 cells were analyzed for each protocol. These cells were obtained from at least five animals. Data from each animal were averaged, and statistical comparisons were made across animals (repeated measures). The specific numbers of animals analyzed in each protocol are provided in the results. Within an age group, there were no significant differences between vehicle control groups for either anesthetic. Accordingly, the data from these control groups were pooled. Data were compared using analysis of variance with repeated measures with age, experimental condition, anesthetic, and concentration as grouping variables. Multiple comparisons were performed using Bonferroni and Scheffé *post hoc* analysis. These *post hoc* analyses were performed across age and experimental condition. $P < 0.05$ was considered significant (two-tailed). Significant differences between 1 and 2 MAC anesthetic are not indicated. All data are expressed as mean ± SD.

Results

Age-related Differences in Anesthetic Effects on Intracellular Ca^{2+} Concentration Responses to Electrical Stimulation

Neonatal and adult cardiac myocytes did not differ in basal $[Ca^{2+}]_i$ (95 ± 8 nM *vs.* 107 ± 11 nM, respectively). There were no significant differences between age groups, anesthetics, or anesthetic concentration in basal $[Ca^{2+}]_i$. The amplitude of the $[Ca^{2+}]_i$ responses to electrical stimulation in neonatal myocytes (389 ± 49 nM) was significantly smaller compared with adults (891 ± 95 nM; $P < 0.05$). Both anesthetics significantly decreased the amplitude of the $[Ca^{2+}]_i$ response, especially in neonates (fig. 2; $P < 0.05$; n = 5), but sevoflurane had a smaller effect compared with halothane.

Age-related Differences in Volatile Anesthetic Effects on Intracellular Ca^{2+} Concentration Responses to BayK8644

BayK8644 potentiated $[Ca^{2+}]_i$ responses to electrical stimulation to a greater extent in neonates (figs. 3A and B; $P < 0.05$; n = 6). Inhibition of the SR and NCX by exposure to ryanodine, thapsigargin, and KBR 7943 decreased the amplitude of the $[Ca^{2+}]_i$ response to electrical stimulation, but to a lesser extent in neonates (fig. 4; n = 5). In the absence of SR and NCX, BayK8644 significantly increased $[Ca^{2+}]_i$ response to electrical stimulation in neonatal myocytes compared with adults (figs. 5A

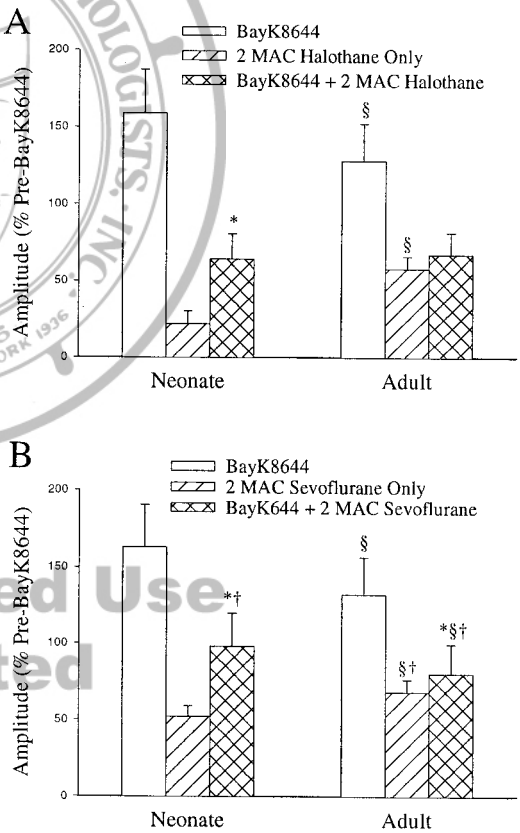

Fig. 3. Interaction between volatile anesthetics and BayK8644 (L-type Ca^{2+} channel agonist). BayK8644 alone increased the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)response to electrical stimulation and blunted the inhibitory effect of halothane (*A*) or sevoflurane (*B*) (only 2 minimum alveolar concentration [MAC] data shown; middle bars in each group represent anesthetic effects without BayK8644). Selected significant differences are highlighted: *effect of adding BayK8644 to anesthetic-induced decrease of the $[Ca^{2+}]_i$ response; §age-related difference; †difference between halothane and sevoflurane. Values are mean ± SD.

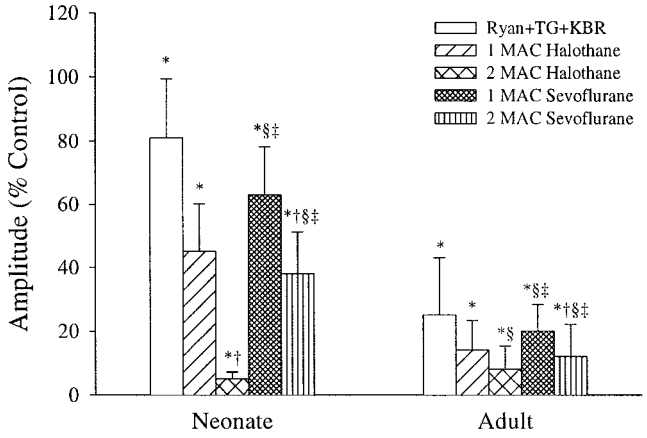

Fig. 4. Volatile anesthetic effects on Ca^{2+} influx channels. Myocytes were exposed to ryanodine, thapsigargin (TG), and KBR 7943 to inhibit sarcoplasmic reticulum (SR) Ca^{2+} release and reuptake, and Na^+–Ca^{2+} exchange (NCX), respectively. The effect of inhibited SR and NCX was greater in adults; however, subsequent exposure to anesthetic decreased the intracellular Ca^{2+} concentration response to electrical stimulation to a greater extent in neonates. *Difference from control; §age-related difference; †difference between 1 and 2 minimum alveolar concentration (MAC); ‡difference between halothane and sevoflurane. Values are mean ± SD.

and B; $P < 0.05$; n = 5 for each age). These data highlight the role of Ca^{2+} influx channels in neonates. Subsequent exposure to anesthetic still resulted in significantly greater decrement in the amplitude of the response in neonates compared with adults (figs. 3A and 4; $P < 0.05$; n = 6). However, at either age, BayK8644 blunted the inhibitory effect of anesthetic. The effects of sevoflurane were generally smaller than those of halothane.

Age-related Differences in Intracellular Ca^{2+} Concentration Response to Caffeine

The transient $[Ca^{2+}]_i$ response in neonatal myocytes (n = 11) was smaller and slower than that of adult myocytes (n = 12; fig. 6, table 1; $P < 0.05$). Even when corrected for SR volume density, the amplitude of the neonatal response (1,983 ± 193 normalized units) was significantly smaller than that of adults (2,678 ± 339 normalized units; $P < 0.05$).

Effect of Volatile Anesthetics on Intracellular Ca^{2+} Concentration Response to Caffeine

Compared with vehicle controls, exposure to anesthetics, especially halothane, decreased the amplitude of the $[Ca^{2+}]_i$ response in both age groups ($P < 0.05$), but to a significantly lesser extent in neonates compared with adults ($P < 0.05$; fig. 6 and table 2). Halothane significantly prolonged increase times in both age groups, but less so in neonates ($P < 0.05$). Compared with the effects on increase time, halothane had considerably greater effects on the decrease time of the $[Ca^{2+}]_i$ response to caffeine, with the effects being several-fold smaller in neonates compared with adults. In general, sevoflurane had minimal to insignificant effects on the $[Ca^{2+}]_i$ responses in neonates.

Effect of Volatile Anesthetics on Sarcoplasmic Reticulum Ca^{2+} Release

Exposure to zero extracellular Ca^{2+}, low Na^+, La^{3+}, CPA, and KBR 7943 did not significantly elevate basal $[Ca^{2+}]_i$. Subsequent exposure to caffeine induced a brisk elevation in $[Ca^{2+}]_i$ (more so in adults) that was more or less sustained. The amplitude of the $[Ca^{2+}]_i$ response was smaller in neonates ($P < 0.05$). Exposure to anesthetics significantly slowed the increase time and blunted the amplitude of the $[Ca^{2+}]_i$ response to caffeine ($P < 0.05$; figs. 7A and B, respectively), but to a greater extent in adults.

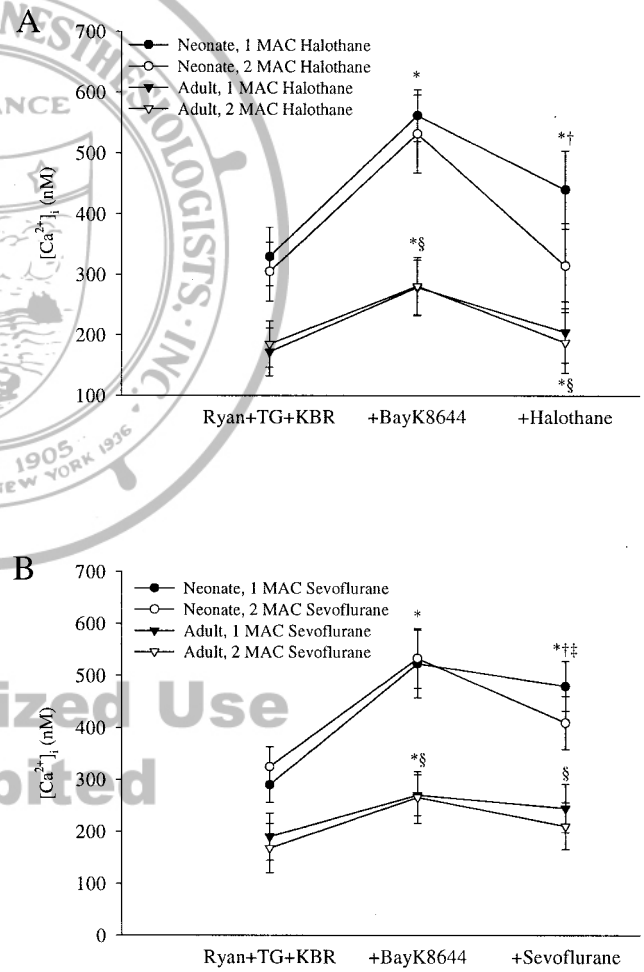

Fig. 5. Comparison of (*A*) halothane *versus* (*B*) sevoflurane interactions with BayK8644-induced elevation of Ca^{2+} influx. When sarcoplasmic reticulum (SR) and Na^+–Ca^{2+} exchange (NCX) were inhibited by ryanodine, thapsigargin (TG), and KBR 7943, the increase in Ca^{2+} influx by BayK8644 was considerably greater in neonates. Subsequent exposure to anesthetic decreased influx to a greater extent in neonates. *Difference from previous condition; §age-related difference; †difference between 1 and 2 minimum alveolar concentration (MAC); ‡difference between halothane and sevoflurane. Values are mean ± SD.

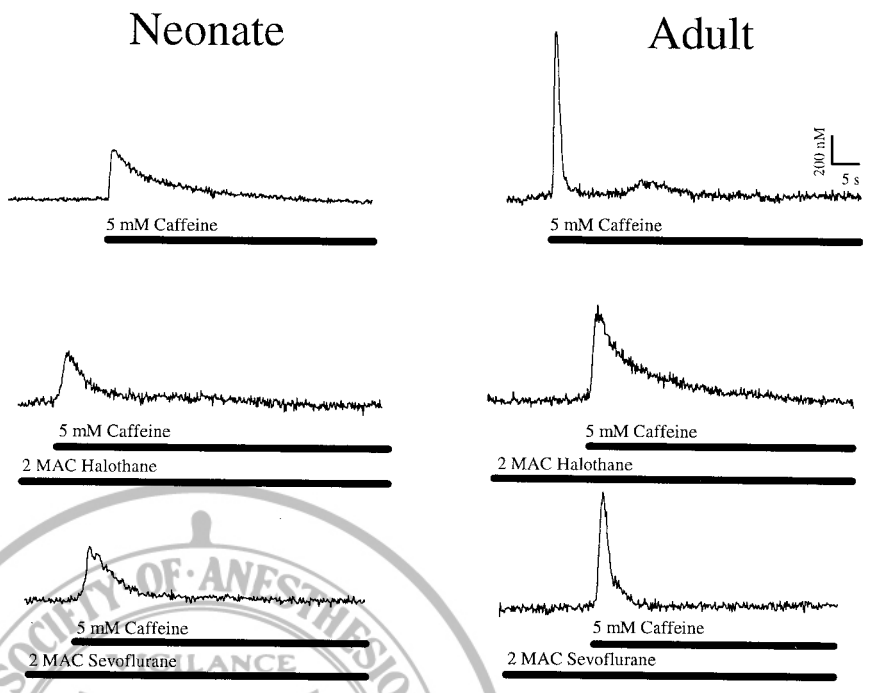

Fig. 6. Age-related differences in volatile anesthetic effects on intracellular Ca^{2+} concentration responses to caffeine. Note the considerably smaller and slower response in neonatal myocytes (*left*) and the smaller effect of both anesthetics in this age group.

Effect of Volatile Anesthetics on Sarcoplasmic Reticulum Ca^{2+} Reuptake

During conditions of zero extracellular Ca^{2+}, low Na^+, La^{3+}, and KBR 7943 (no CPA), the response to caffeine was qualitatively similar to that in figure 6. The decrease time, representing SERCA activity, was significantly slower in neonates ($P < 0.05$). Preexposure to anesthetic significantly slowed the decrease time of subsequent caffeine responses, especially in the adult (fig. 7C).

Estimation of Sarcoplasmic Reticulum Volume Density

Cross-sections stained for RyR channels displayed a relatively less-organized SR in neonate compared with adult cardiac muscle, as expected. Accordingly, the SR volume density of the neonatal muscle, estimated using either cross-sections or single cells, was significantly less than that of adult muscle (6.3 ± 2.3% *vs.* 28.4 ± 3.7% of the cell area; $P < 0.05$).

Effects of Volatile Anesthetics on Influx Mode of Na^+–Ca^{2+} Exchange in Neonates versus *Adults*

Na^+ loading did not significantly change resting $[Ca^{2+}]_i$. Rapid reintroduction of $[Ca^{2+}]_o$, with simultaneous removal of extracellular Na^+ concentration ($[Na^+]_o$), resulted in a rapid and monotonic increase in $[Ca^{2+}]_i$. Introduction of normal Tyrode solution resulted in a plateau. The influx rate of NCX (calculated from the slope of the steepest portion of the ascending curve) ranged from 74.4 to 132.3 nM/s in neonates compared with 32.7 to 78.3 nM/s in adults (pooled control data; n = 10 for each age). Both halothane and sevoflurane slowed NCX-mediated Ca^{2+} influx to a greater extent in neonatal myocytes (fig. 8; $P < 0.05$; n = 5 for each concentration and age). In adults, slowing of influx was comparable between the two anesthetics.

Volatile Anesthetic Effects on Efflux Mode of Na^+–Ca^{2+} Exchange in Neonates versus *Adults*

Simultaneous inhibition of SERCA and NCX resulted in a rapid increase in $[Ca^{2+}]_i$ followed by a slow decline

Table 1. Age-related Differences in $[Ca^{2+}]_i$ Response to Caffeine

	Neonate	Adult
Amplitude (nM)	199 ± 60	741 ± 75*
Rise time (ms/nM)	13.1 ± 7.1	4.0 ± 2.1*
Fall time (ms/nM)	91.1 ± 31.2	12.3 ± 12.5*

Values are represented as mean ± SD.

* indicates significant age-related difference ($P < 0.05$).

Table 2. Effect of Volatile Anesthetics on $[Ca^{2+}]_i$ Responses to Caffeine

		Neonate	Adult
Amplitude	Control	100 ± 11	124 ± 13
	Halothane	67 ± 11*	44 ± 10*†
	Sevoflurane	102 ± 10	91 ± 9*†
Rise time	Control	121 ± 12	75 ± 13
	Halothane	165 ± 13*	287 ± 14*†
	Sevoflurane	95 ± 9	249 ± 13*†
Fall time	Control	107 ± 13	76 ± 9
	Halothane	192 ± 13*	603 ± 31*†
	Sevoflurane	86 ± 9	111 ± 11

Values are % of first caffeine exposure. Only two minimum alveolar concentration (MAC) values are reported (mean ± SD).

* indicates significant difference from control; † indicates significant age-related difference ($P < 0.05$).

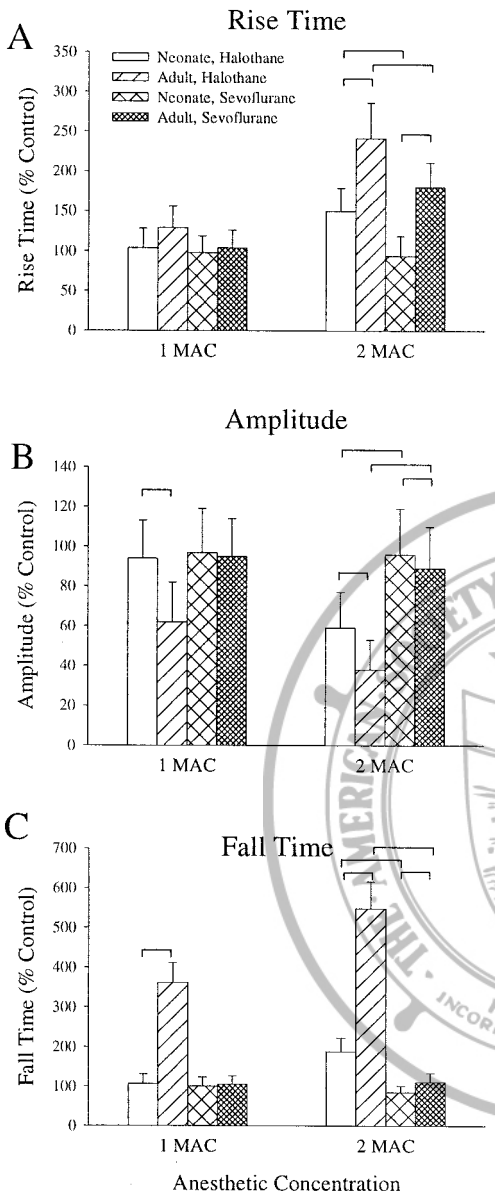

Fig. 7. Age-related differences in the effect of volatile anesthetics on sarcoplasmic reticulum (SR) Ca^{2+} release and reuptake. The SR was functionally isolated by inhibiting Ca^{2+} influx (see Methods). To examine SR Ca^{2+} release, reuptake was reversibly inhibited using cyclopiazonic acid (CPA), and cells were exposed to caffeine. The caffeine and CPA were washed, and the protocol was repeated in the presence of anesthetic. The increase time of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) response to caffeine (*A*) represented the rate of SR Ca^{2+} release and the amplitude (*B*) SR Ca^{2+} content. To examine reuptake, the SR was isolated but CPA was not added. The decrease time of the $[Ca^{2+}]_i$ response represented the rate of SR Ca^{2+} reuptake. Overall, volatile anesthetic effects were more pronounced in adults, rather than in neonates. Brackets indicate significant differences. Values are mean ± SD.

(most likely because of the PMCA, which was not specifically inhibited in this protocol). Rapid reintroduction of $[Na^+]_o$ resulted in a steep decrease in $[Ca^{2+}]_i$. The efflux rate of NCX (calculated from the steepest portion of the descending curve) ranged from 55.4 to 95.2 nM/s in neonates *versus* 18.5 to 36.2 nM/s in adults (pooled control data; n = 10). In the presence of anesthetic, reactivation of NCX resulted in significantly slower efflux in neonatal myocytes, both in absolute terms (fig. 9A; $P < 0.05$; n = 5) and when normalized for "peak" $[Ca^{2+}]_i$ (fig. 9B; $P < 0.05$[18]). Compared with the neonate, anesthetic effects on efflux in adults were smaller (figs. 9C and D; $P < 0.05$). Overall, sevoflurane had a smaller effect on efflux rates in both age groups ($P < 0.05$).

Volatile Anesthetic Effects on Na^+ Dependence of Na^+-Ca^{2+} Exchange in Neonates versus *Adults*

Na^+ Dependence of Influx. Because the time for Na^+ loading was fixed, the extent of Na^+ loading was assumed to be proportional to $[Na^+]_o$. The actual Na^+ concentration in the cell was not determined. Decreasing Na^+ loading resulted in comparable slowing of influx in both neonates and adults (fig. 10; $P < 0.05$). Exposure to halothane resulted in a further, concentration-dependent slowing of NCX-mediated Ca^{2+} influx, especially in neonates (figs. 10A and B; $P < 0.05$; n = 5), such that 2 MAC halothane completely inhibited influx at 35 mM Na^+. Sevoflurane produced less slowing of influx compared with halothane, at least in neonates (figs. 10C and D; $P < 0.05$ for sevoflurane effects only; n = 5).

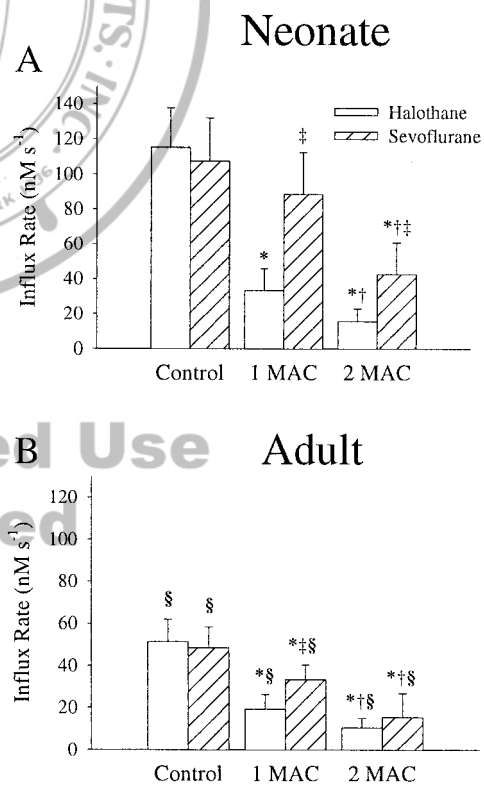

Fig. 8. Age-related differences in the effect of volatile anesthetics on Na^+–Ca^{2+} exchange (NCX)-mediated Ca^{2+} influx. Both anesthetics inhibited Ca^{2+} influx to a greater extent in neonatal myocytes (*A*) compared with adults (*B*). *Difference from control; §age-related differences; †difference between 1 and 2 minimum alveolar concentration (MAC); ‡difference between halothane and sevoflurane. Values are mean ± SD.

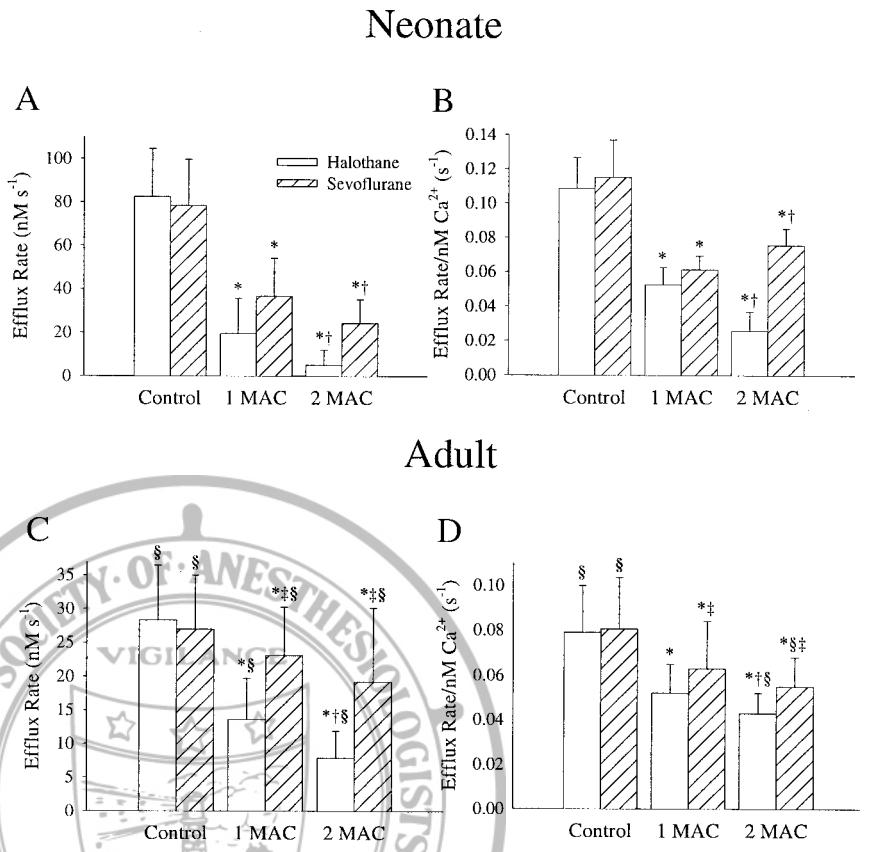

Fig. 9. Age-related difference in the effect of volatile anesthetics on Na^+–Ca^{2+} exchange (NCX)-mediated Ca^{2+} efflux. Both anesthetics inhibited absolute Ca^{2+} efflux (*A*), as well as rates normalized for intracellular Ca^{2+} concentration (*B*) to a greater extent in neonatal myocytes compared with adults (*C* and *D*). *Difference from control; §age-related differences; †difference between 1 and 2 minimum alveolar concentration (MAC); ‡difference between halothane and sevoflurane. Values are mean ± SD.

Na^+ Dependence of Efflux. Decreasing $[Na^+]_o$ slowed NCX-mediated efflux to a greater extent in neonates (fig. 11; $P < 0.05$). The confounding influence of different "peak" $[Ca^{2+}]_i$ values on efflux rate was avoided by making measurements only in cells displaying comparable "peak" $[Ca^{2+}]_i$ values. Halothane further slowed efflux at each Na^+ concentration, especially in neonates (figs. 11A and B; $P < 0.05$; n = 5). Overall, sevoflurane produced less slowing of NCX-mediated efflux in both age groups for every Na^+ concentration (figs. 11C and D; $P < 0.05$; n = 5).

Discussion

The current study demonstrates that volatile anesthetics decrease $[Ca^{2+}]_i$ to a greater extent in neonates by greater inhibition of Ca^{2+} channels and NCX, rather than inhibition of SR Ca^{2+} release and reuptake. Given a greater role for Ca^{2+} influx channels and NCX in $[Ca^{2+}]_i$ regulation of the neonatal heart, inhibition of these mechanisms by volatile anesthetics is likely to have a greater impact on cardiac function at that age.

Methodologic Issues

A potential concern with separate techniques for dissociating cardiac myocytes (Langendorf *vs.* trypsin-based mechanical dissociation) is whether the techniques themselves explain some of the age-related differences in volatile anesthetic effects. However, given the lack of difference in basal $[Ca^{2+}]_i$ and the response to caffeine, it is unlikely that these issues, even if present, were relevant in the current study. "Freshly" dissociated neonatal cardiac myocytes express only 25% of the total Ca^{2+} current density of that in "freshly" dissociated adult myocytes.[8] Our data in 1-day-old neonatal myocytes are consistent and suggest that the dissociation technique alone cannot account for the observed age-related differences in $[Ca^{2+}]_i$ regulation.

Although fluo-3 is a nonratiometric $[Ca^{2+}]_i$ indicator, in several studies[18,19] we found relatively small variations in basal $[Ca^{2+}]_i$ across myocytes from different coverslips and animals, underlining the reliability of the empirical Ca^{2+} calibration technique using this dye. A potential limitation of fluo-3, especially relating to adult cardiac myocytes, is its relative lack of sensitivity at extremely high $[Ca^{2+}]_i$ values. Although it is possible that peak $[Ca^{2+}]_i$ responses may have been underestimated, the results and conclusions of the study are unlikely to be affected because comparisons were made within the same cell before and during anesthetic exposure.

Pharmacologic techniques rather than electrical stimulation were used to inhibit or activate NCX during defined conditions of $[Ca^{2+}]_i$, $[Ca^{2+}]_o$, $[Na^+]_i$, and $[Na^+]_o$ because several studies, including the current one, have demonstrated that anesthetics inhibit both

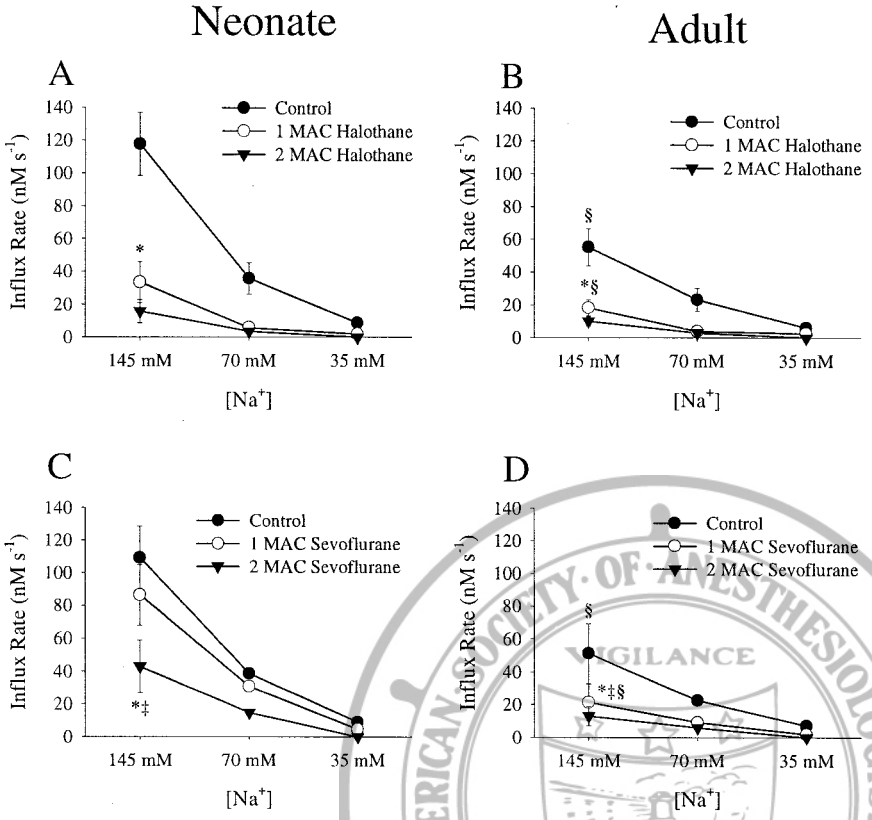

Fig. 10. Age-related differences in the effect of volatile anesthetics on the Na^+ dependence of Na^+–Ca^{2+} exchange (NCX)-mediated influx. The rate of Ca^{2+} influx was determined after Na loading cells with one of three different extracellular Na^+ concentrations for a fixed period of time. Both anesthetics blunted the relation between Na^+ concentration and Ca^{2+} influx rate in neonates (*A* and *C*) as well as adults (*B* and *D*), with the effect being greater in neonates. *Difference from control; §age-related differences; ‡difference between halothane and sevoflurane. Values are mean ± SD.

Ca^{2+} channels[12,14,23] and SR Ca^{2+} release–reuptake,[15–17] which would have influenced the $[Ca^{2+}]_i$ response to electrical stimulation. Furthermore, to isolate anesthetic effects on NCX using electrical stimulation, agents such as nifedipine or ryanodine would only have abolished the $[Ca^{2+}]_i$ responses.

In the NCX efflux protocol, we did not specifically inhibit the PMCA, which may have accounted for the slow decline in $[Ca^{2+}]_i$ observed even in the absence of $[Na^+]_o$. However, the contribution of PMCA to the overall results are unlikely to be significant, because the PMCA has a 10-fold higher affinity for Ca^{2+} but a considerably slower turnover rate compared with NCX[24] (evidenced by large change in slope of the $[Ca^{2+}]_i$ profile after reintroduction of $[Na^+]_o$).

The current study used MAC values from adult rats for comparison across age groups. Similar to clinical observations, it is possible that MAC values of halothane and sevoflurane in neonatal rats may be increased compared with adult rat values. Regardless, the fact that the lower adult MAC values used in the current study resulted in greater inhibition of $[Ca^{2+}]_i$ regulatory mechanisms only highlights greater anesthetic sensitivity of the neonatal myocardium.

Volatile Anesthetic Effects on Ca^{2+} Influx Channels

Consistent with previous results in adult hearts,[12–14] we found that halothane and sevoflurane both inhibit the opening of L-type channels by BayK8644. However, studies with halothane and isoflurane have found no differences in potency in inhibition of Ca^{2+} influx. We found that, unlike isoflurane, which has effects comparable to halothane,[12] sevoflurane had a much smaller impact on Ca^{2+} influx in adult cardiac myocytes compared with halothane. Differences in relative anesthetic potency may be related to individual lipid solubilities and potential changes in channel protein configuration. Furthermore, volatile anesthetics may differ in their relative impact on other mechanisms that regulate Ca^{2+} influx channels, such as cyclic nucleotides and protein kinases.

Inasmuch as Ca^{2+} influx plays a greater role in $[Ca^{2+}]_i$ regulation of the neonatal heart, this is a potential mechanism underlying greater myocardial depression produced by volatile anesthetics. Inhibition of Ca^{2+} influx channels can reduce $[Ca^{2+}]_i$ directly by reducing the contribution to total $[Ca^{2+}]_i$ and indirectly by preventing Ca^{2+}-induced Ca^{2+} release (CICR). Previous studies have suggested that the contribution of Ca^{2+} influx *per se* to total $[Ca^{2+}]_i$ in the neonatal heart may not be substantial, because Ca^{2+} channel protein and peak current densities are actually lower.[9,11,25,26] However, the surface area of the neonatal myocyte is also smaller than that of the adult. Accordingly, even though absolute Ca^{2+} current may be small, it may have a significant contribution to total $[Ca^{2+}]_i$. Regardless, inhibition of Ca^{2+} influx by volatile anesthetics will decrease its contribution to total

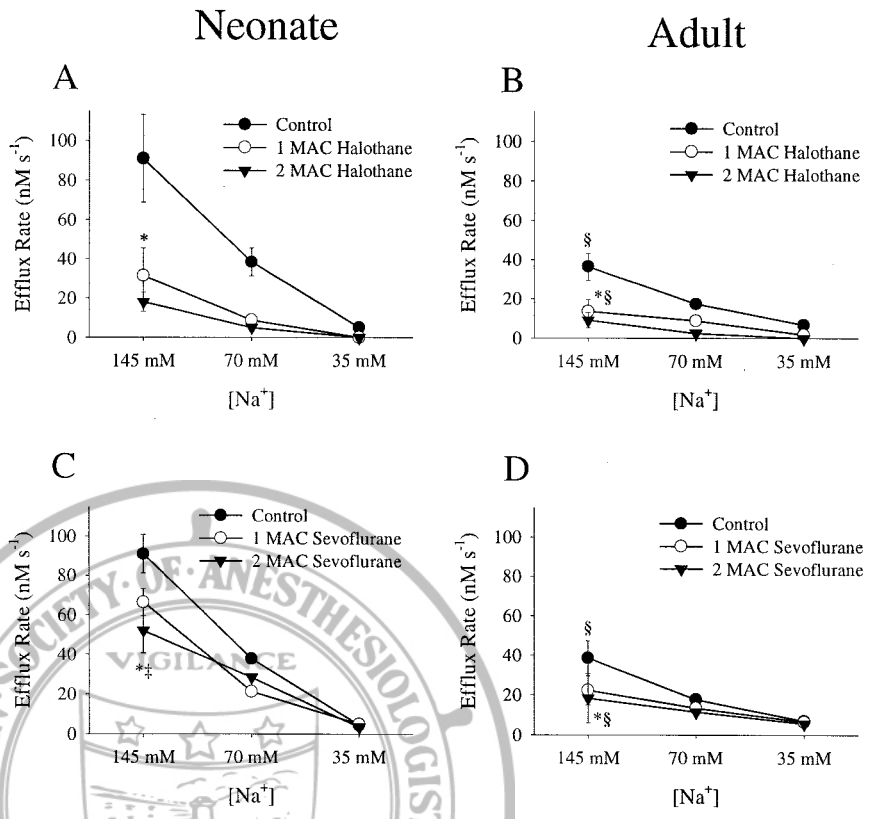

Fig. 11. Age-related differences in the effect of volatile anesthetics on the Na^+-dependence of Na^+–Ca^{2+} exchange (NCX)-mediated efflux. The rate of Ca^{2+} efflux was determined after reactivating efflux with one of three different extracellular Na^+ concentrations. Both anesthetics blunted the relation between Na^+ concentration and Ca^{2+} efflux rate in neonates (*A* and *C*) as well as adults (*B* and *D*), with the effect being greater in neonates. *Difference from control; §age-related differences; ‡difference between halothane and sevoflurane. Values are mean ± SD.

$[Ca^{2+}]_i$ and may further interfere with CICR, leading to the same overall effect, *i.e.*, greater reduction in $[Ca^{2+}]_i$.

Volatile Anesthetic Effects on the Sarcoplasmic Reticulum

Previous studies have demonstrated that the neonatal SR is functionally immature.[27] In accordance, the current study found that, compared with the adult, neonatal cardiac myocytes have significantly smaller SR Ca^{2+} stores that are released during activation. During these conditions, volatile anesthetics (halothane more so than sevoflurane) inhibited SR Ca^{2+} release to a greater extent in adult cardiac myocytes, rather than neonatal myocytes, indicating that greater sensitivity of neonatal myocardium to anesthetics cannot be explained by greater effects at the level of the SR.

In adult cardiac myocytes, the pronounced effects of halothane on the $[Ca^{2+}]_i$ response to caffeine are consistent with several previous studies.[15–17] The general consensus has been that anesthetics increase SR Ca^{2+} "leak" through RyR channels, thus depleting SR Ca^{2+}. Only a few studies have examined age-related differences in volatile anesthetic effects at the level of the SR and have also interpreted the results as greater SR Ca^{2+} leak in the neonate.[1] Nonetheless, given the smaller and immature SR, it is still unlikely that increased SR Ca^{2+} leak can explain the greater sensitivity of neonatal cardiac myocytes to volatile anesthetics.

Volatile anesthetics have also been shown to decrease SR Ca^{2+} content, independent of an effect on RyR channels.[28] Our results using halothane are generally consistent with these previous reports. In addition, we found that sevoflurane, chemically closer to isoflurane, produces a smaller effect on the $[Ca^{2+}]_i$ response to caffeine, a finding consistent with the study by Davies *et al.*[29] in adult rat heart. Therefore, it is possible that differences in volatile anesthetic effects on SR Ca^{2+} content underlie potency differences for myocardial depression. However, as with SR Ca^{2+} leak, the smaller SR makes it unlikely that decreased SR Ca^{2+} content can explain greater myocardial depression in neonates.

Whether volatile anesthetics have an effect on SERCA is a particularly relevant issue in the neonatal heart, where relaxation is already limited by the lower rate, number, and efficiency of SERCA protein.[30] Some studies have reported a decrease in reuptake,[31] whereas others have reported either no effect[32] or increased reuptake.[33] A potential problem with directly attributing the effects of volatile anesthetics to SERCA is that these effects are critically dependent on both pH and adenosinde triphosphate concentration. Furthermore, even when pH is controlled,[34] it is difficult to isolate the effects of NCX from SERCA in decreasing $[Ca^{2+}]_i$. However, in the current study, we inhibited NCX-mediated Ca^{2+} efflux by using low extracellular Na^+ and still found that halothane had a profound effect on the decrease time of the $[Ca^{2+}]_i$ response to caffeine, at least in the adult. Accordingly, these data clearly demonstrate that volatile anesthetics inhibit SERCA. The overall effect of this inhibition on $[Ca^{2+}]_i$ regulation will then depend on the relative

role of SERCA and may thus be of less consequence in the neonate.

Role of Na^+-Ca^{2+} Exchange in Intracellular Ca^{2+} Concentration Regulation

It is now well-recognized that NCX-mediated Ca^{2+} efflux plays a key role in relaxation of the adult heart,[4,5,35] albeit with species differences in its relative contribution to $[Ca^{2+}]_i$ regulation.[36] Some studies in the rat[36,37] have suggested a role for NCX-mediated Ca^{2+} influx in CICR. The results of the current study, as well as those from a recently published study,[18] show that NCX-mediated influx during conditions of inhibited Ca^{2+} channels and SR, and NCX-mediated efflux during conditions of inhibited SERCA, are both demonstrable in adult rat cardiac myocytes.

In the current study, we found that the rate of NCX-mediated Ca^{2+} influx is higher in the neonate. Based on the finding that, at peak action potential, Ca^{2+} influx can occur even when Ca^{2+} channels are inactivated, Wetzel *et al.*[10] suggested that NCX-mediated Ca^{2+} influx likely plays a physiologic role in the neonatal heart. Furthermore, sarcolemmal vesicles isolated from newborn rabbit ventricle have been shown to exhibit greater Na^+-dependent Ca^{2+} uptake[38] as well as NCX mRNA[39] and protein content[38] (determined immunologically) compared with adult animals. Artman *et al.*[40] determined that NCX current density in neonatal rabbit myocytes is greatest perinatally and reaches lower adult values by the third postnatal week. These results support a key role for NCX in neonatal cardiac $[Ca^{2+}]_i$ regulation. Furthermore, NCX-mediated Ca^{2+} efflux may then be key to relaxation of the neonatal heart, evidenced by a higher rate of NCX-mediated Ca^{2+} efflux.

Studies have shown that the direction of Ca^{2+} flux *via* NCX depends on membrane potential itself, $[Na^+]_i$, $[Na^+]_o$, $[Ca^{2+}]_o$, or $[Ca^{2+}]_i$.[4,41,42] We previously demonstrated the relation between $[Na^+]_i$ and rate of NCX-mediated Ca^{2+} influx and that between $[Na^+]_o$ and Ca^{2+} efflux in adult rat cardiac myocytes.[18] In this study, we found no significant age-related differences in the relation between Ca^{2+} influx or efflux and $[Na^+]_i$ and $[Na^+]_o$.

Volatile Anesthetic Effects on Na^+-Ca^{2+} Exchange in Neonates versus *Adults*

Detailed studies on anesthetic and NCX interactions have been limited by the lack of specific inhibitors, as well as concurrent anesthetic effects on other $[Ca^{2+}]_i$ regulatory mechanisms. Interactions between volatile anesthetics and NCX need to be interpreted with caution. During contraction, NCX inhibition should decrease the inward Ca^{2+} current and produce negative inotropy, adding to the decrease in $[Ca^{2+}]_i$ produced by inhibition of Ca^{2+} influx channels. On the other hand, inhibition of NCX during diastole should decrease the outward Ca^{2+} current, delay Ca^{2+} efflux, and produce positive inotropy. Accordingly, the net effect of NCX inhibition would depend on several factors: (1) the relative role of NCX in elevating and decreasing $[Ca^{2+}]_i$; (2) the duration of NCX activation during a single cardiac cycle, anesthetic effects on this duration, and thus the overall contribution to total $[Ca^{2+}]_i$; (3) the degree of compensation by other mechanisms involved in $[Ca^{2+}]_i$ regulation. These issues may need to be addressed in studies examining NCX-mediated Ca^{2+} currents during the cardiac cycle using electrophysiologic techniques.

There are no published studies on age-related differences on volatile anesthetic interactions with NCX. In adult rat cardiac myocytes, halothane, isoflurane, and enflurane all completely inhibit NCX.[43] In a recently published study on adult rat cardiac myocytes, we found that both halothane and sevoflurane inhibit NCX-mediated Ca^{2+} influx as well as efflux.[18] In the current study, we found that both anesthetics produce greater inhibition of NCX in neonatal cardiac myocytes. Such an effect may decrease $[Ca^{2+}]_i$ to a greater extent in neonates that are already more dependent on this mechanism.

In the recently published study on adult rat cardiac myocytes,[18] we were the first to report that both halothane and sevoflurane blunt the relation between NCX-mediated Ca^{2+} influx and $[Na^+]_i$, as well as between NCX-mediated Ca^{2+} efflux and $[Ca^{2+}]_i$ and $[Na^+]_o$. In the current study, we found that the extent of blunting of these relations by anesthetics is greater in neonatal myocytes, especially for Ca^{2+} influx. These data also support the idea that inhibition of NCX is one mechanism by which anesthetics produce greater myocardial depression in neonates.

Volatile Anesthetics and Influx Mode of Na^+-Ca^{2+} Exchange

The little data on the inhibitory effects of volatile anesthetics on influx mode NCX in cardiac muscle are limited to the adult.[43] In adult rat cardiac myocytes exposed to zero $[Na^+]_o$, Blanck *et al.*[44] found no effect of halothane on NCX and speculated that NCX sensitivity to volatile anesthetics was reduced during these conditions. On the other hand, in our previous study, we also examined NCX-mediated Ca^{2+} influx during conditions of zero $[Na^+]_o$[18] and found that volatile anesthetics have a proportionately greater inhibitory effect on influx mode of NCX compared with efflux. This discrepancy may be related to the fact that our results represent anesthetic effects on the maximum rates of NCX-mediated influx or efflux. It is possible that during the cardiac cycle, combined anesthetic effects on NCX rate as well as on duration of NCX activation (if any) may lead to a different scenario in terms of NCX contribution to total $[Ca^{2+}]_i$.

In the current study, we further demonstrate that NCX-mediated Ca^{2+} influx is decreased to a greater extent in

neonates by anesthetics. Given the smaller role of the SR and the relatively slow kinetics of Ca^{2+} influx channels, NCX-mediated influx may be key to neonatal cardiac contraction. Accordingly, anesthetic inhibition may have a disproportionately greater effect on contractility in the neonate. We also found that anesthetics produce considerable alterations in the relation between $[Na^+]_i$ and NCX-mediated influx, especially in neonates. Previous studies[41,42] have shown that $[Na^+]_i$ activation of NCX-mediated influx is sigmoidal with a half-maximal activation of 15–25 mM, easily achieved with Na^+ loading in our protocols. It is possible that volatile anesthetics interfere with Na^+ binding, which is regulated by $[Ca^{2+}]_i$,[24] perhaps to a greater extent in neonates. However, even if comparable between age groups, such inhibition may have greater functional impact in the neonate.

Volatile Anesthetics and Efflux Mode of Na^+–Ca^{2+} Exchange

The efflux mode of NCX is arguably more important in cardiac function, especially in the neonate. There are currently very little published data on anesthetic effects on this aspect of NCX.[18] We had reported that anesthetics decrease efflux, blunt the normal positive correlations between $[Ca^{2+}]_i$ and efflux rate, as well as $[Na^+]_o$ and efflux.[4,42] We now demonstrate that volatile anesthetics produce significantly greater rightward shift in the Na^+ dependence of NCX-mediated efflux in neonatal myocytes compared with the adult. In a recent review, Blaustein and Lederer[4] speculated that the binding of three Na^+ ions to two binding sites is essential for proper functioning of NCX-mediated Ca^{2+} efflux. As with influx, age- and anesthetic-related differences in efflux at different $[Na^+]_o$ values may be related to competitive interference with Na^+ binding. Another potential factor is the energy level of the cell, which is known to influence the Na^+ affinity of NCX.[4]

Clinical Relevance

The overall effect of volatile anesthetics on the $[Ca^{2+}]_i$ profile in cardiac myocytes will be determined by the relative contributions of different regulatory mechanisms. If Ca^{2+} influx channels and NCX play greater roles in the neonate for elevating $[Ca^{2+}]_i$, their inhibition may well overcome any positive inotropy produced by simultaneous inhibition of efflux mode NCX and SERCA. Volatile anesthetics also affect compensatory neural mechanisms of cardiac control,[45] which may be particularly important in neonates, where the sensitivity of the myocardium to neural stimulation may already be lower (*e.g.*, β-adrenoceptor sensitivity and adenylyl cyclase activities are lower[46]).

Compared with humans and some other species such as rabbits, the adult rat heart is more dependent on SR Ca^{2+} for contraction, whereas the neonatal rat heart is clearly dependent on plasma membrane Ca^{2+} fluxes. The relative roles of these mechanisms in neonates of other species are still under investigation. It is possible that even with comparable quantitative effects on Ca^{2+} regulatory mechanisms, age-related differences in anesthetic sensitivity may arise purely from the fact that different mechanisms are relatively more important. However, we demonstrated that anesthetic effects, especially on plasma membrane Ca^{2+} fluxes, are both qualitatively and quantitatively different between neonates and adults. Accordingly, even in species such as humans, where plasma membrane Ca^{2+} fluxes are more important, age-related differences in anesthetic sensitivity may still arise from greater inhibition of L-type Ca^{2+} channels and NCX, consistent with our hypothesis.

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