

Isoflurane Reduction of Carbachol-evoked Cytoplasmic Calcium Transients Is Dependent on Caffeine-sensitive Calcium Stores

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Background: **Many muscarinic functions are relevant to anesthesia, and alterations in muscarinic activity affect the anesthetic/analgesic potency of various drugs. Volatile anesthetics have been shown to depress muscarinic receptor function, and inhibition of the muscarinic signaling pathway alters the minimal alveolar anesthetic concentration of inhaled anesthetics. The purpose of this investigation was to determine in a neuronal cell which source of Ca^{2+} underlying the carbachol-evoked transient increase in cytoplasmic Ca^{2+} was reduced by isoflurane.**

Methods: **Experiments were performed at 37°C on continuously perfused monolayers of human neuroblastoma SH-SY5Y cells using Fura-2 as the cytoplasmic Ca^{2+} indicator. Carbachol (1 mM) was applied to evoke a transient increase in cytoplasmic Ca^{2+}.**

Results: **Isoflurane (1 mM) reduces the carbachol-evoked transient increase in cytoplasmic Ca^{2+}, and this isoflurane action is eliminated when the cells are continuously stimulated with 200 mM KCl or pretreated with 10 mM caffeine or 200 μM ryanodine.**

Conclusions: **Isoflurane reduction of the carbachol-evoked transient increase in cytoplasmic Ca^{2+} requires full caffeine-sensitive Ca^{2+} stores and Ca^{2+} release from the caffeine-sensitive stores through the ryanodine-sensitive Ca^{2+} release channels. The results indicate that isoflurane interferes with a muscarinic Ca^{2+} signaling through a mechanism downstream from the muscarinic receptors.**

MANY muscarinic functions are relevant to anesthesia and alterations in muscarinic activity affect the anesthetic/analgesic potency of various drugs. Brain stem muscarinic signaling modulates the level of consciousness,[1] whereas cortical muscarinic signaling affects memory and learning.[2,3] Spinal muscarinic receptors inhibit glutamate release[4] and enhance γ-aminobutyric acid release.[5] Muscarinic agonists have been reported to enhance antinociceptive effects, and this enhancement is blocked by pretreatment with M1, M2, or M3 muscarinic receptor antagonists or with antisense inhibition of M1 receptors.[6–8] However, there are also reports indicating that muscarinic block enhances the analgesic/anesthetic action of various drugs, and that some of these drugs induce muscarinic block.[9,10] Halothane and isoflurane have been shown to depress muscarinic receptor function,[11–14] and inhibition of the muscarinic signaling pathway has been shown to have variable effects on the minimal alveolar anesthetic concentration of inhaled anesthetics.[15] Therefore, the role of muscarinic receptors in analgesia/anesthesia seems to be complex and can either augment or attenuate the anesthetic potency of the volatile anesthetics.

Muscarinic activation leads to a transient elevation of cytoplasmic Ca^{2+} ($[Ca^{2+}]_{cyt}$) through Ca^{2+} release from intracellular stores. We investigated the action of volatile anesthetics on the $[Ca^{2+}]_{cyt}$ transients evoked by a muscarinic agonist, carbachol, in human SH-SY5Y cells. We previously reported that halothane reduced the carbachol-evoked $[Ca^{2+}]_{cyt}$ transient in a concentration-dependent manner, and that sustained stimulation with KCl eliminated this effect of halothane.[16] The Ca^{2+} contributing to the carbachol-evoked $[Ca^{2+}]_{cyt}$ transient seems to derive from both inositol tri-phosphate-sensitive and caffeine-sensitive Ca^{2+} stores.[16] The purpose of this investigation was to determine which source of Ca^{2+} underlying the carbachol-evoked $[Ca^{2+}]_{cyt}$ transient was reduced by isoflurane.

Materials and Methods

Cell Culture and Solutions

Experiments were performed on monolayers of SH-SY5Y human neuroblastoma cells (originally provided by June Biedler, Ph.D., Sloan-Kettering Institute for Cancer Research, Rye, NY at the providing time; Dr. Biedler is currently a Distinguished Resident Scientist, Fordham University, Bronx, NY), passages 60–94. Cells were cultured in RPMI1640 medium with L-glutamine, supplemented with penicillin (50 U/ml), streptomycin (50 μg/ml), and 12% fetal bovine serum at 37°C, in a humidified atmosphere containing 5% CO_2. All cell culture components were Gibco BRL products purchased from Life Technologies (Rockville, MD). Cells were plated on glass coverslips (25-mm diameter) at a density of approximately $2–4 \times 10^4$ cells/ml (2 ml/Petri dish) and used when they formed a confluent monolayer (*i.e.*, between 8–20 days after plating). The bath solution was a HEPES buffer containing (in mM) 140 NaCl, 5 KCl, 5 $NaHCO_3$, 10 HEPES, 1 $MgCl_2$, 1.5 $CaCl_2$, 1 ATP, and 10 glucose (pH 7.4). All experiments

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Received from the Anesthesiology Department, New York University School of Medicine, New York, New York.Submitted for publication January 31, 2003. Accepted for publication May 12, 2003. Supported by grant No. RO1GM50686 from the National Institutes of Health, Bethesda, Maryland (to Dr. Blanck). Presented in part at the annual meeting of the American Society of Anesthesiologists, Orlando, Florida, October 12–16, 2002.

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were performed at 37°C, and the temperature was controlled with a Dual Heater controller TC-344A and an inline heater SH-27B (Warner Instruments Inc., Hamden CT). All the solutions and instruments were prewarmed to 37°C before being used. The exchange of the solution was done using a manifold, with its common outlet placed in the entrance of the heater inline. The solutions containing [200 mM] KCl (EM-Science), [10 mM] caffeine, [1 mM] carbachol, or [200 μM] ryanodine were prepared using the HEPES buffer. Saturated isoflurane (Ohmeda Caribe Inc., Guayana, PR) solutions were prepared in HEPES buffer 24 h in advance in gas-tight containers and diluted to the final concentration (1 mM) immediately before used as previously described.[16]

Ca^{2+} Measurements

SH-SY5Y cells were loaded with the fluorescent Ca^{2+} indicator Fura-2[17] by incubating the cells on coverslips in the culture medium containing 5 μM of the acetoxymethyl ester of the dye (Fura-2 AM; Molecular Probes, Eugene, OR) for 30 min under culture conditions.[16] After loading, cells were gently washed three times with the HEPES buffer, and the coverslips were placed into the perfusion chamber and perfused (250 μl/min) for 30 min with the buffer at 37°C to allow their equilibration with the experimental environment before being exposed to the various drugs. The bath solution alone and containing the individual or combination of drugs was perfused at 250 μl/min for various periods as indicated in the figure legends.

The perfusion chamber was set on an inverted microscope (DIAPHOT 300; Nikon, Melville, NY) equipped with a ×40 oil-immersion objective (N.A.1.30, Nikon). The microscope was attached to a high-speed multiwavelength illuminator (DeltaRAM V; Photon Technology International, Inc., PTI, Lawrenceville, NJ). The excitation wavelengths for Fura-2 (340 nm and 380 nm) were alternately (every 0.02 s) generated with a monochromator. The emitted fluorescence (from the alternated excitation at 340 and 380 nm) from 15–20 cells was filtered with the fluorescence barrier filter BA 515 nm, collected with a photomultiplier (PMT01–710, Photon Technology International), and digitized at 50 Hz.

Data Analysis

Data collection and analysis was carried out with Felix (version 1.42a, Photon Technology International) and Clampfit (Pclamp 8; Axon Instruments, Foster City, CA) software. For each treatment (corresponding to data in each figure), experiments were performed under different conditions on sister cultures (same plating day) and on three to five culture sets (different plating days). The averaged traces shown in the figures were obtained by lining up the peak values for the evoked $[Ca^{2+}]_{cyt}$ transients. Unless otherwise indicated, the areas were obtained over a period of 300 s starting from the onset of the carbachol-evoked $[Ca^{2+}]_{cyt}$ transient on each trace. In the figures, the data represent the ratio (in protocols and steady states) or the Δ ratio (for peak and areas) of the emission of Fura-2 at 515 nm obtained with 340 and 380 nm excitations (ratio 340/380). Control steady-state levels were measured just before the addition of any compound, and the new steady-state level after the addition of a particular compound was measured just before the addition of the subsequent compound. Steady-state levels measurements are expressed as ratio (340/380) values.

Statistical Analysis

Comparison between different groups was performed using the unpaired or paired two-tailed *t* test with Sigma software (Jandel Scientific Corp., San Rafael, CA).

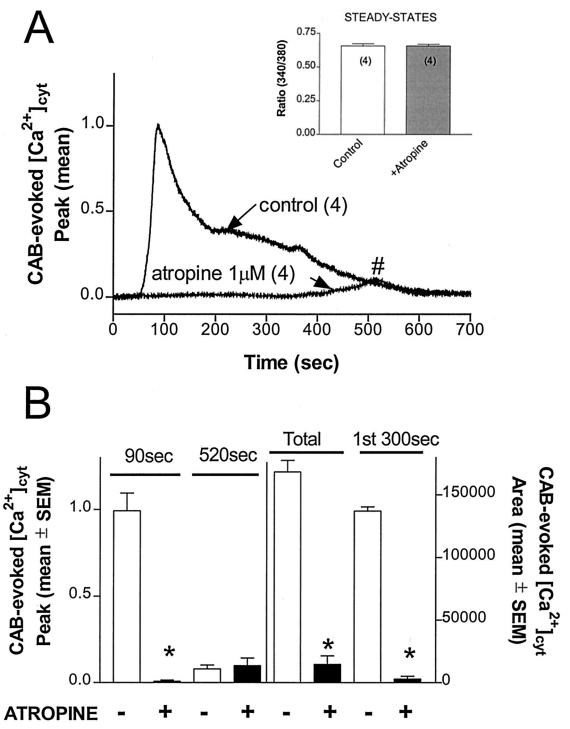

Fig. 1. Atropine blocks the carbachol-evoked $[Ca^{2+}]_{cyt}$ transient. Cells were exposed to 1 mM carbachol (CAB) for 2 min in the absence or presence of 1 μM atropine. (*A*) The averaged carbachol-evoked $[Ca^{2+}]_{cyt}$ transients in the absence and presence of atropine. *Number sign* indicates the location of the carbachol-evoked $[Ca^{2+}]_{cyt}$ peak in the presence of atropine. The *inset* shows the steady-state levels of $[Ca^{2+}]_{cy}$ in the absence and presence of atropine. (*B*) The maximal response at the time of the carbachol-evoked $[Ca^{2+}]_{cyt}$ peak in the absence of atropine (*90 sec*) and in the presence of atropine (*520 sec*) (*left*); and the area values during either the entire (*Total*) or the first 300 s (*1st 300 sec*) of the carbachol-evoked $[Ca^{2+}]_{cyt}$ transient (*right*). *Asterisk* indicates a statistically significant difference ($P < 0.0001$, paired *t* test) between control and atropine conditions.

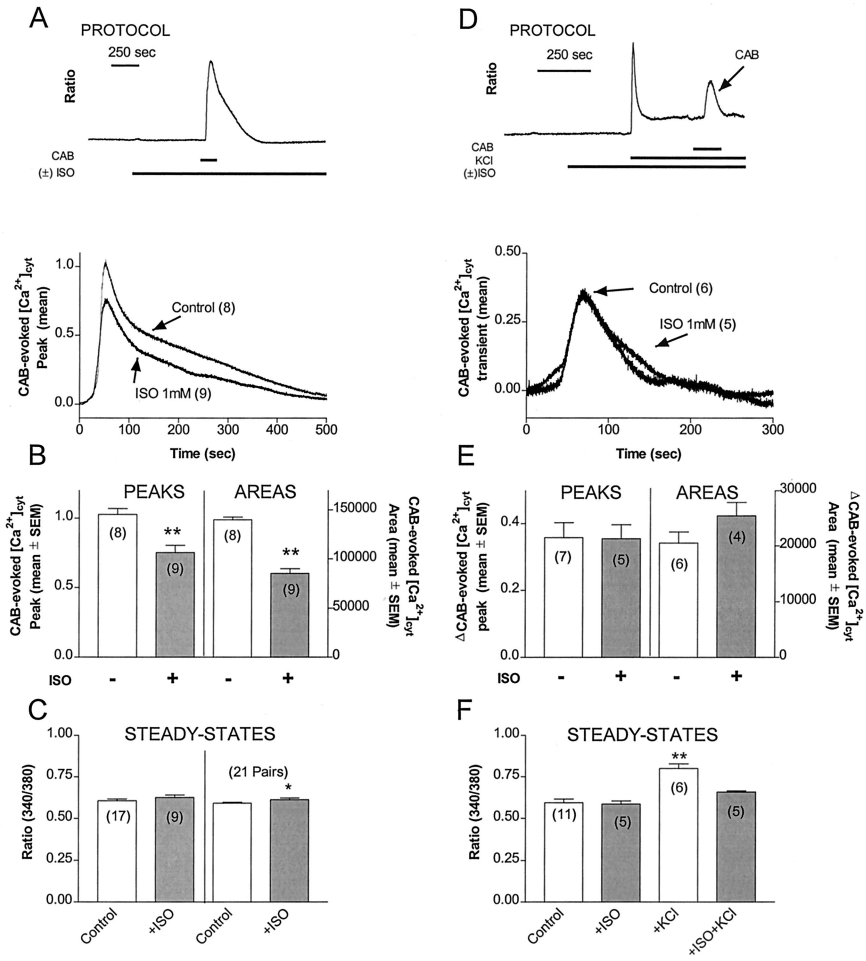

Fig. 2. Isoflurane reduces the carbachol-evoked $[Ca^{2+}]_{cyt}$ transient, and this reduction is eliminated by continuous exposure to KCl. (*A, B*) Cells were exposed to 1 mM carbachol (CAB) for 2 min in the absence or presence of 1 mM isoflurane (ISO). The application of isoflurane was started 10 min before the carbachol stimulation. (*A*) The averaged carbachol-evoked $[Ca^{2+}]_{cyt}$ transients in the absence and presence of isoflurane. (*B*) The mean peak and area values of the carbachol-evoked $[Ca^{2+}]_{cyt}$ transient in the absence and presence of isoflurane. (*C*) The corresponding $[Ca^{2+}]_{cyt}$ steady-state levels (*left*) and the paired $[Ca^{2+}]_{cyt}$ steady-state levels (control and isoflurane measurements performed on the same cells) obtained from data shown in figures 2 and 4, in the absence and presence of isoflurane. (*D, E*) Cells were treated as described for panels *A* and *B* but in the presence of 200 mM KCl. The application of KCl was started 5 min before the carbachol stimulation. (*D*) The averaged carbachol-evoked $[Ca^{2+}]_{cyt}$ transients in the presence of KCl and in the absence and presence of isoflurane. (*E*) The mean peak and area values for the carbachol-evoked $[Ca^{2+}]_{cyt}$ transient in the presence of KCl and in the absence and presence of isoflurane. (*F*) The corresponding $[Ca^{2+}]_{cyt}$ steady-state levels. *Asterisks* indicate a statistically significant difference ($P < 0.001$, unpaired *t* test; *$P < 0.005$, paired *t* test) between control and a given group.**

Results

Atropine Blocked the Carbachol-evoked $[Ca^{2+}]_{cyt}$ Transient

Atropine (1 μM), a muscarinic blocker, completely (>99%) blocked the main peak of the carbachol-evoked $[Ca^{2+}]_{cyt}$ transient (fig. 1, *90 sec*). Carbachol activated a small nonmuscarinic increase in $[Ca^{2+}]_{cyt}$ that was activated later than the main muscarinic-evoked $[Ca^{2+}]_{cyt}$ increase (fig. 1A) and it represented 7.4% of the total area of the carbachol-evoked $[Ca^{2+}]_{cyt}$ transient (fig. 1B, *Total*). In the following sections, the area measurements for the carbachol-evoked $[Ca^{2+}]_{cyt}$ transients were performed over the first 300 s after the onset of the carbachol-evoked $[Ca^{2+}]_{cyt}$ transient; during this period, the nonmuscarinic component represents less than 2% of the area (fig. 1B, *1st 300 sec*).

Isoflurane Reduced the Carbachol-evoked $[Ca^{2+}]_{cyt}$ Transient and Continuous Stimulation with KCl Eliminated the Isoflurane-mediated Reduction of the Carbachol-evoked $[Ca^{2+}]_{cyt}$ Transient

Figure 2 shows the averaged response for the carbachol-evoked $[Ca^{2+}]_{cyt}$ transient before and after the exposure to 1 mM isoflurane. Isoflurane significantly reduced the peak and the area of the carbachol-evoked $[Ca^{2+}]_{cyt}$ transient (fig. 2A and B). Continuous stimulation with 200 mM KCl reduced the carbachol-evoked $[Ca^{2+}]_{cyt}$ response (fig. 2D *vs.* A). However, if the cells were continuously stimulated with 200 mM KCl, 1 mM isoflurane did not produce additional changes in the peak height or the area of the carbachol-evoked $[Ca^{2+}]_{cyt}$ response (fig. 2D and E). Isoflurane produced a slight increase in the steady-state level of $[Ca^{2+}]_{cyt}$ that only became significant when the paired data were considered (fig. 2C). However, continuous KCl stimulation produced a large increase in the $[Ca^{2+}]_{cyt}$ steady-state level, and this increase was attenuated by isoflurane (fig. 2F). Isoflurane also reduced the peak and area values of the KCl-evoked $[Ca^{2+}]_{cyt}$ transient (fig. 3). We previously reported that isoflurane had a biphasic concentration-dependent effect on the KCl-evoked $[Ca^{2+}]_{cyt}$ transient, increasing the transient at low concentrations and decreasing it at higher concentrations.[16] In our previous study, 1 mM isoflurane produced no significant change compared with the control value,[16] but in this study it decreased the KCl-evoked $[Ca^{2+}]_{cyt}$ transient, suggesting that a sensitivity increase in the isoflurane effect mediates the reduction of the KCl-evoked $[Ca^{2+}]_{cyt}$ transient. This difference may have resulted from the different conditions under which the

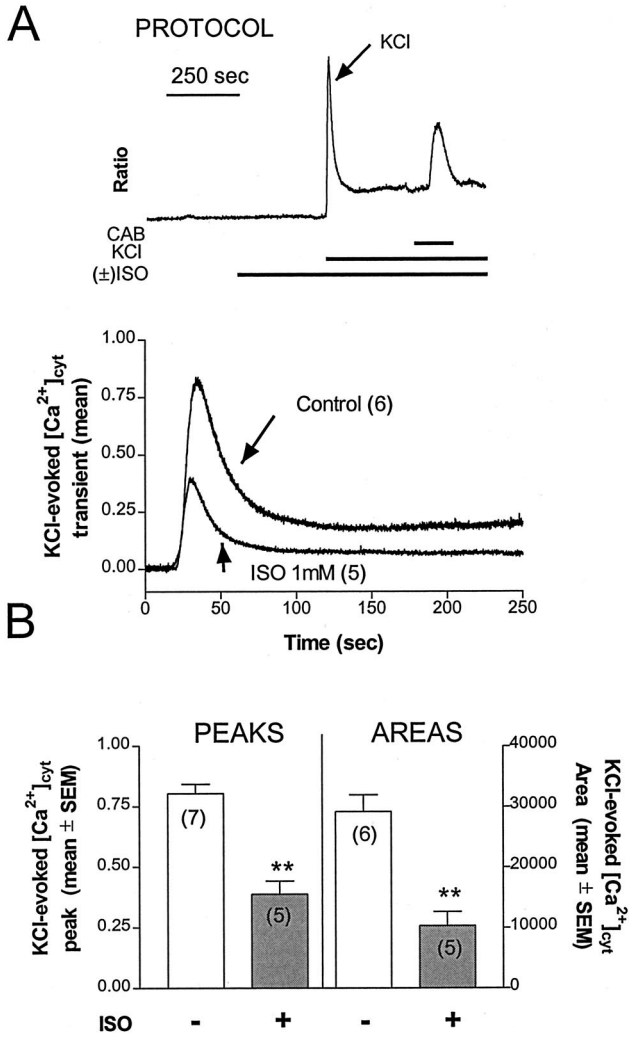

Fig. 3. Isoflurane reduces the KCl-evoked $[Ca^{2+}]_{cyt}$ transient. The conditions are the same as described in figure 1B. (*A*) The averaged KCl-evoked $[Ca^{2+}]_{cyt}$ transients in the absence and presence of 1 mM isoflurane (ISO). (*B*) The mean peak and area values for the KCl-evoked $[Ca^{2+}]_{cyt}$ transients in the absence and presence of isoflurane. The corresponding steady-state levels of $[Ca^{2+}]_{cyt}$ are shown in figure 2F. *Asterisks* indicate a statistically significant difference ($P < 0.001$, unpaired *t* test) between control and isoflurane conditions.

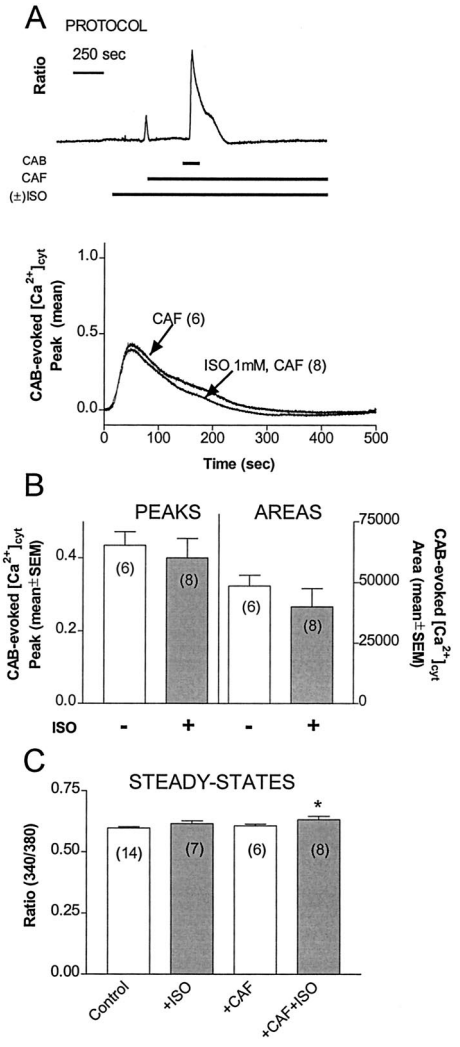

Fig. 4. Caffeine treatment eliminates isoflurane-mediated reduction of the carbachol-evoked $[Ca^{2+}]_{cyt}$ transient. Cells were exposed to 1 mM carbachol (CAB) for 2 min in the presence of 10 mM caffeine (CAF) and in the absence or presence of 1 mM isoflurane (ISO). The application of isoflurane was started 10 min before the carbachol stimulation, and the application of caffeine was started 5 min before the carbachol stimulation. (*A*) The averaged carbachol-evoked $[Ca^{2+}]_{cyt}$ transient in the absence and presence of isoflurane. (*B*) The mean peak and area values for the carbachol-evoked $[Ca^{2+}]_{cyt}$ transient in the absence and presence of isoflurane. (*C*) The corresponding steady-state levels of $[Ca^{2+}]_{cyt}$. *Asterisk* indicates a statistically significant difference ($P < 0.05$, unpaired *t* test) between control and a given group.

cells were grown (high-density confluent multilayer[16] *vs.* monolayer). However, in both studies it is clear that continuos treatment with KCl eliminated the inhibition by both halothane[16] and isoflurane (present study) of the carbachol-evoked $[Ca^{2+}]_{cyt}$ response.

Isoflurane-mediated Reduction of the Carbachol-evoked $[Ca^{2+}]_{cyt}$ Transient Required Full Caffeine-sensitive Ca^{2+} Stores

We then investigated whether a direct depletion of the caffeine-sensitive Ca^{2+} stores could also eliminate the isoflurane-mediated reduction of the carbachol-evoked $[Ca^{2+}]_{cyt}$ transient. We previously showed that the carbachol-evoked $[Ca^{2+}]_{cyt}$ transient involves Ca^{2+} release mostly from the inositol tri-phosphate-sensitive Ca^{2+} store but also from caffeine-sensitive stores.[16] In this study, we found that pretreatment with 10 mM caffeine reduced the carbachol-evoked $[Ca^{2+}]_{cyt}$ response (fig. 4A *vs.* fig. 2A, *controls*). In the presence of caffeine, isoflurane did not produce an additional reduction of the carbachol-evoked $[Ca^{2+}]_{cyt}$ response (fig. 4), suggesting that this action of isoflurane requires a full caffeine-sensitive Ca^{2+} store. The steady-state level of $[Ca^{2+}]_{cyt}$ was not affected by isoflurane or caffeine when added individually, and it only displayed a slightly significant increase when they were applied together (fig. 4C).

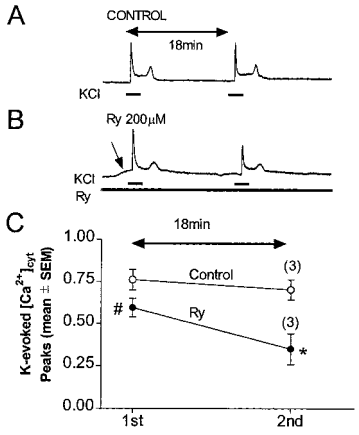

Fig. 5. Ryanodine decreases the KCl-evoked $[Ca^{2+}]_{cyt}$ transient. Cells were stimulated two consecutive times with 200 mM KCl in the absence (*A*) and presence (*B*) of 200 μM ryanodine (Ry). The application of ryanodine was started 5 min before the first KCl stimulation, and KCl was applied for 2 min. The time interval between the first and second KCl pulse was 18 min. When cells were exposed to ryanodine, the initial response was an increase in the $[Ca^{2+}]_{cyt}$ (indicated with an *arrow*). (*C*) The mean peak values for the first and the second KCl-evoked $[Ca^{2+}]_{cyt}$ transients in the absence (*open circles*) and presence (*filled circles*) of ryanodine. *Asterisk* and *number sign* indicate a statistically significant difference ($P < 0.02$ and $P < 0.05$, respectively; unpaired *t* test) between control and ryanodine groups.

Isoflurane-mediated Reduction of the Carbachol-evoked $[Ca^{2+}]_{cyt}$ Transient Required Ca^{2+} Release from the Caffeine-sensitive Store through the Ryanodine-sensitive Ca^{2+} Release Channels

To test whether the isoflurane-mediated reduction of the carbachol-evoked $[Ca^{2+}]_{cyt}$ required Ca^{2+} release from caffeine-sensitive store through ryanodine-sensitive Ca^{2+} release channels, we blocked ryanodine-sensitive Ca^{2+} release channels by exposing the cells to 200 μM ryanodine. Because activation of Ca^{2+} release channels is required to obtain maximal channel block by ryanodine,[18,19] the following protocol was developed. Cells were pretreated for 5 min with ryanodine, exposed to a short KCl pulse (to activate ryanodine-sensitive Ca^{2+} release channels), and after 18 min (time required for Ca^{2+} refilling of intracellular Ca^{2+} stores) exposed to carbachol. The level of channel block was determined by measuring the ryanodine action on two consecutive KCl-evoked $[Ca^{2+}]_{cyt}$ transients (fig. 5). When cells were exposed to ryanodine, the initial response was an increase in the $[Ca^{2+}]_{cyt}$ (fig. 5, *arrow*), which has been reported to reflect activation of ryanodine-sensitive Ca^{2+} channels.[19,20] Ryanodine showed only a small blocking effect on the first KCl-evoked $[Ca^{2+}]_{cyt}$ response (fig. 5C, *1st*), even when cells were exposed to ryanodine for 48 h (data not shown); however, ryanodine strongly blocked the second KCl-evoked $[Ca^{2+}]_{cyt}$ response (fig. 5C, *2nd*). In contrast to the continuous exposure to KCl (fig. 2D), the KCl pulse did not eliminate the isoflurane-mediated reduction of the carbachol-evoked $[Ca^{2+}]_{cyt}$ transient (fig. 6); although the isoflurane-mediated area reduction was significant, the peak reduction did not reach a significant level (fig. 6A and B). Ryanodine pretreatment did not significantly decrease the carbachol-evoked $[Ca^{2+}]_{cyt}$ response (peak or area) (fig. 6A and B, *control vs.* fig. 6D and E, *control*), in contrast to continuous exposure to KCl (fig. 2B, *control vs.* E, *control*) or caffeine treatment (fig. 4B, *control vs.* fig. 2B, *control*). However, ryanodine pretreatment still eliminated the isoflurane-mediated reduction of the carbachol-evoked $[Ca^{2+}]_{cyt}$ transient (fig. 6D and E), suggesting that the isoflurane-mediated reduction of the carbachol-evoked $[Ca^{2+}]_{cyt}$ transient requires Ca^{2+} release from the caffeine-sensitive Ca^{2+} stores through ryanodine-sensitive Ca^{2+}-release channels. The steady-state level of $[Ca^{2+}]_{cyt}$ was slightly increased after the KCl pulse (fig. 6C) or after ryanodine pretreatment (fig. 6F); under both of these conditions, the addition of isoflurane did not produce additional changes in the steady-state level of $[Ca^{2+}]_{cyt}$ (fig. 6C and F).

Discussion

The purpose of this study was to determine the intracellular source of the portion of the carbachol-evoked $[Ca^{2+}]_{cyt}$ transient that was reduced by volatile anesthetics. Our previous work suggested that this portion of Ca^{2+} was derived from the caffeine-sensitive Ca^{2+} store.[16] We have demonstrated in this study that the portion of the carbachol-evoked $[Ca^{2+}]_{cyt}$ transient that is reduced by halothane is also reduced by isoflurane, that it arises from the caffeine-sensitive Ca^{2+} store, and that it is released from the caffeine-sensitive store through ryanodine-sensitive Ca^{2+} release channels.

The role of muscarinic receptors in analgesia/anesthesia is complex because, as discussed previously, both activation and blockade of muscarinic receptors have been shown to be analgesic. Our results indicate that, at the concentrations used, isoflurane blocks only part of the carbachol-evoked $[Ca^{2+}]_{cyt}$ response, apparently at site that is distal to the muscarinic receptor. This isoflurane-sensitive component may be contributing to the anesthetic potency of isoflurane. The previously reported variable effects of muscarinic blockers on the minimum alveolar concentration of inhaled anesthetics could reflect differences in the magnitude of this isoflurane-sensitive component in various brain/spinal regions, where the muscarinic blockers were applied.

The experiments with caffeine show that the isoflurane-mediated blocking action on the carbachol-evoked $[Ca^{2+}]_{cyt}$ transient requires that caffeine-sensitive Ca^{2+} stores are not depleted. We show that ryanodine at 200 μM, which is known to be a maximal blocking concentration,[19,21] reduces the KCl-evoked $[Ca^{2+}]_{cyt}$ response by 50% without reducing the carbachol-evoked $[Ca^{2+}]_{cyt}$ response, and eliminates the isoflurane-medi-

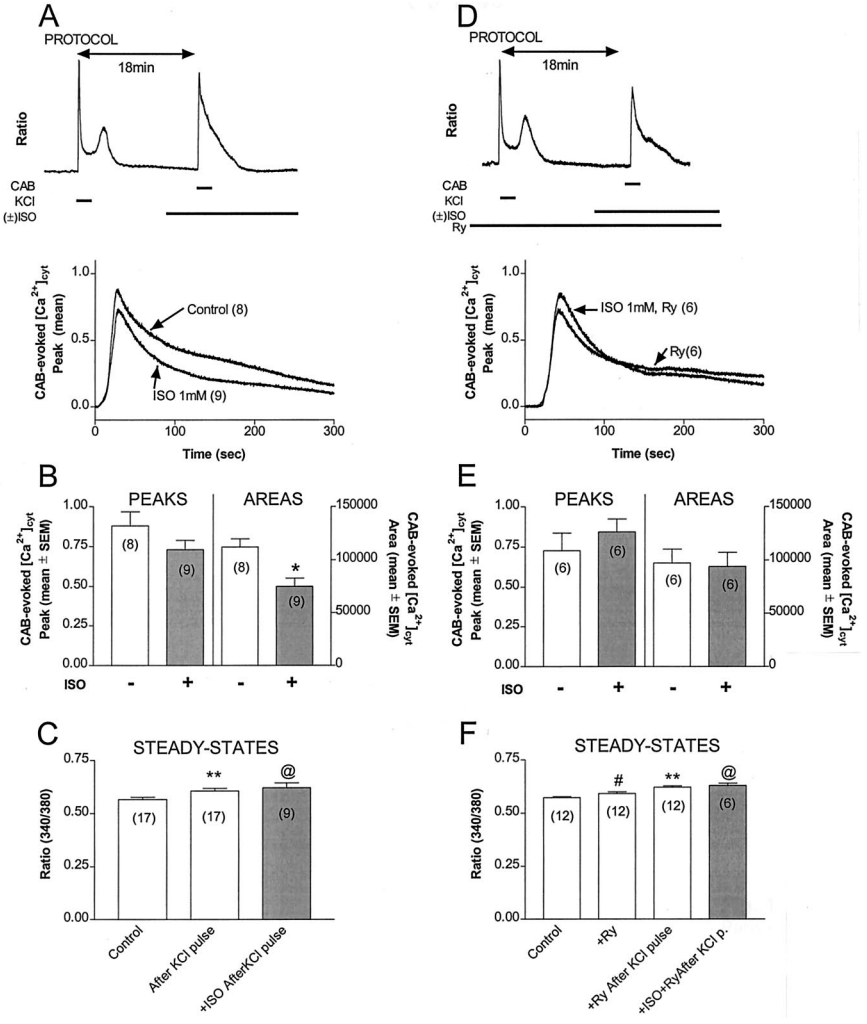

Fig. 6. Ryanodine treatment eliminates the isoflurane-mediated reduction of the carbachol-evoked $[Ca^{2+}]_{cyt}$ transient. Cells were exposed to 1 mM carbachol (CAB) for 2 min after a transient (2 min) stimulation with 200 mM KCl, in the absence (*A–C*) or presence (*D–F*) of 200 μM ryanodine (Ry) and in the absence or presence of 1 mM isoflurane (ISO), respectively. The application of the KCl stimulation was done 18 min before the carbachol stimulation. Ryanodine application was started 5 min before the KCl stimulation, and the isoflurane application was started 10 min before the carbachol stimulation. The averaged carbachol-evoked $[Ca^{2+}]_{cyt}$ transients (*A*), and the mean peak and area values for the carbachol-evoked $[Ca^{2+}]_{cyt}$ transients (*B*), following the KCl pulse are shown in the absence and presence of isoflurane. In the presence of ryanodine, the averaged carbachol-evoked $[Ca^{2+}]_{cyt}$ transients (*D*), and the mean peak and area values for the carbachol-evoked $[Ca^{2+}]_{cyt}$ transients (*E*), following the KCl pulse are shown in the absence and presence of ISO. (*C, F*) The corresponding $[Ca^{2+}]_{cyt}$ steady-state levels. Symbols indicate a statistically significant difference between control and a given group (*$P < 0.005$, @$P < 0.02$ unpaired *t* test; #$P < 0.005$, **$P < 0.0001$, paired *t* test).

ated blocking effect on the carbachol-evoked $[Ca^{2+}]_{cyt}$ transient. This finding indicates that Ca^{2+} release through ryanodine channels is required for the isoflurane-mediated blocking action on the carbachol-evoked $[Ca^{2+}]_{cyt}$ transient. There are several possible explanations for the mechanism by which isoflurane and halothane may reduce the carbachol-evoked $[Ca^{2+}]_{cyt}$ transient. One possibility is that isoflurane and halothane actually block the opening of the ryanodine-sensitive Ca^{2+} release channel; however, this appears unlikely because halothane,[22] and perhaps isoflurane,[16] actually enhance the opening of the ryanodine-sensitive Ca^{2+} release channel. A second possibility is that isoflurane decreases the level of Ca^{2+} in the caffeine-sensitive Ca^{2+} stores by enhancing Ca^{2+} release through ryanodine-sensitive Ca^{2+} channels. However, further experiments are required to determine whether the isoflurane action on the carbachol-evoked $[Ca^{2+}]_{cyt}$ transient results from a direct or indirect action of isoflurane on the ryanodine-sensitive Ca^{2+} channels. As an indirect action, Ca^{2+} is released from the *caffeine*-sensitive stores and shuttled to the inositol tri-phosphate-sensitive stores through volatile-anesthetic-sensitive transporters (*e.g.*, similar to the binding of Ca^{2+}, entering through L-type Ca^{2+} channels, to calmodulin),[23,24] or Ca^{2+} could be released from the caffeine-sensitive stores and inhibits a volatile-anesthetic-sensitive channel (*e.g.*, capacitative channels at the plasma membrane). In addition, Ca^{2+} released through the ryanodine-sensitive Ca^{2+} channels could be in close vicinity to Ca^{2+} transport systems that are activated by volatile anesthetics such as the sarco/endoplasmic reticulum Ca^{2+}-pumping adenosine triphosphatase.[25] At this time there is no evidence to implicate any of these or other possible mechanisms. In SH-SY5Y cells, muscarinic agonists increase $[Ca^{2+}]_{cyt}$ and the level of inositol triphosphate,[26] and in smooth muscle cells halothane decreases the carbachol-evoked $[Ca^{2+}]_{cyt}$ increase and reduces inositol tri-phosphate production.[27] Therefore, isoflurane may inhibit the carbachol-evoked $[Ca^{2+}]_{cyt}$ transient by reducing the inositol tri-phosphate production. However, a decrease in the inositol tri-phosphate production or any direct isoflurane action on muscarinic receptors (even if they take place) would not explain our observations. If the latter actions were responsible for the isoflurane-mediated decrease in the carbachol evoked $[Ca^{2+}]_{cyt}$ transient, the isoflurane effect under

the various conditions studied (continuous KCl, ryanodine or caffeine treatment) would still be expected.

In summary, this study shows that isoflurane interferes with muscarinic-evoked $[Ca^{2+}]_{cyt}$ transients through a mechanism downstream from the muscarinic receptors. Isoflurane reduces the carbachol-evoked $[Ca^{2+}]_{cyt}$ transient. The carbachol-evoked $[Ca^{2+}]_{cyt}$ transient is dependent on Ca^{2+} derived from both inositol tri-phosphate- and caffeine-sensitive Ca^{2+} stores. Reduction of the carbachol-evoked $[Ca^{2+}]_{cyt}$ transient by isoflurane is eliminated either when the caffeine-sensitive Ca^{2+} store is depleted or when the release of Ca^{2+} from the caffeine-sensitive Ca^{2+} store is blocked by ryanodine. The relationship between the modulation of Ca^{2+} homeostasis by volatile anesthetics and the mechanisms of anesthesia remains unclear and must be examined in more complex systems.

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