Anesthesiology
1998; 89:202–14

Thiopental Alters Contraction, Intracellular Ca^{2+}, and pH in Rat Ventricular Myocytes

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Background: **Myocardial contractility is regulated by intracellular concentration of free Ca^{2+} ($[Ca^{2+}]_i$) and myofilament Ca^{2+} sensitivity. The objective of this study was to elucidate the direct effects of thiopental on cardiac excitation–contraction coupling using individual, field-stimulated ventricular myocytes.**

Methods: **Freshly isolated rat ventricular myocytes were loaded with the Ca^{2+} indicator, fura-2, and placed on the stage of an inverted fluorescence microscope in a temperature-regulated bath. $[Ca^{2+}]_i$ (340/380 ratio) and myocyte shortening (video-edge detection) were monitored simultaneously in individual cells field-stimulated at 0.3 Hz. Amplitude and timing of myocyte shortening and $[Ca^{2+}]_i$ were compared before and after addition of thiopental. Intracellular *p*H was measured with the *p*H indicator, BCECF (500/440 ratio). Real-time uptake of Ca^{2+} into isolated sarcoplasmic reticulum vesicles was measured using fura-2 free acid in the extravesicular compartment. One hundred thirty-two cells were studied.**

Results: **Field stimulation increased $[Ca^{2+}]_i$ from 85 ± 10 nM to 355 ± 22 nM (mean ± SEM). Myocytes shortened by 10% of resting cell length (127 ± 5 μm). Times to peak $[Ca^{2+}]_i$ and shortening were 139 ± 6 and 173 ± 7 msec, respectively. Times to 50% recovery for $[Ca^{2+}]_i$ and shortening were 296 ± 6 and 290 ± 6 ms, respectively. Addition of thiopental (30–1,000 μM) resulted in dose-dependent decreases in peak $[Ca^{2+}]_i$ and myocyte shortening. Thiopental altered time to peak and time to 50% recovery for $[Ca^{2+}]_i$ and myocyte shortening and inhibited the rate of uptake of Ca^{2+} into isolated sarcoplasmic reticulum vesicles. Thiopental did not, however, alter the amount of Ca^{2+} released in response to caffeine in sarcoplasmic reticulum vesicles or intact cells. Thiopental (100 μM) increased intracellular *p*H and caused an upward shift in the dose–response curve to extracellular Ca^{2+} for shortening, with no concomitant effect on peak $[Ca^{2+}]_i$. These effects were abolished by ethylisopropyl amiloride, an inhibitor of Na^+–H^+ exchange.**

Conclusion: **Thiopental has a direct negative inotropic effect on cardiac excitation–contraction coupling at the cellular level, which is mediated by a decrease in $[Ca^{2+}]_i$. Thiopental also increases myofilament Ca^{2+} sensitivity *via* alkalinization of the cell, which may partially offset its negative inotropic effect. (Key words: Intravenous anesthetic; myofilament sensitivity; negative inotrope; sarcoplasmic reticulum.)**

USE of thiopental for induction of anesthesia is sometimes associated with hypotension and cardiac depression.[1,2] Because of concomitant changes in preload, afterload, baroreflex activity, and central nervous system activity after induction, the direct effects of thiopental on cardiac contractility are difficult to ascertain *in vivo*.[3] *In vitro* studies provide a more direct approach for examining the specific effects of thiopental on myocardial contractility. Thiopental causes myocardial depression in isolated perfused hearts[4] and isometrically contracting papillary muscles.[5–11] Inhibition of transsarcolemmal Ca^{2+} influx[6,8,11–14] and uptake[11,15] of Ca^{2+} by, or release[9,11] of Ca^{2+} from, the sarcoplasmic reticulum (SR) have been suggested as potential cellular mechanisms of action of thiopental. Other reports have indicated, however, that thiopental inhibits K^+ currents,[12,16] increases duration of action potential,[11,16] and enhances myofilament Ca^{2+} sensitivity[6] but does not alter uptake of Ca^{2+} by isolated SR vesicles.[17] Therefore, thiopental could exert direct effects on the cardiomyocyte through multiple sites of action. The functional consequences and mechanisms of action of thiopental at the cellular level have not been established, however.

This study evaluated the direct effects of thiopental on excitation–contraction coupling in individual freshly isolated rat ventricular myocytes. This experi-

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Received from the Center for Anesthesiology Research and the Department of Molecular Cardiology, The Cleveland Clinic Foundation, Cleveland, Ohio. Submitted for publication September 16, 1997. Accepted for publication March 24, 1998. Supported in part by grants HL-38291 and HL-40361 from the National Heart, Lung and Blood Institute; Dr. Kanaya was supported by Professor Akiyoshi Namiki, M.D., Ph.D., Department of Anesthesiology, Sapporo Medical University School of Medicine, Sapporo, Japan. Presented in part at the annual meeting of the American Society of Anesthesiologists, October 18–22, 1997, San Diego, California.

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mental model allowed us to simultaneously measure changes in the amplitude and timing of intracellular Ca^{2+} transients and myocyte shortening. We tested the hypothesis that thiopental would reduce myocyte shortening *via* a decrease in concentration of intracellular free Ca^{2+} ($[Ca^{2+}]_i$). We also investigated the effects of thiopental on the handling of Ca^{2+} by the SR, myofilament Ca^{2+} sensitivity, and intracellular *p*H (pH_i).

Methods and Materials

Preparation of Ventricular Myocytes

Isolated adult ventricular myocytes from rat hearts were obtained as previously described.[18] The hearts were excised, cannulated *via* the aorta, attached to a modified Langendorff perfusion apparatus, and perfused with oxygenated (95% O_2/5% CO_2) Krebs-Henseleit buffer (37°C) containing the following (in mM): 118 NaCl, 4.8 KCl, 1.2 $MgCl_2$, 1.2 KH_2PO_4, 1.2 $CaCl_2$, 37.5 $NaHCO_3$, and 16.5 dextrose, *p*H 7.35. After a 5-min equilibration period, the perfusion buffer was changed to a Ca^{2+}-free Krebs-Henseleit buffer (120 ml) containing 30 mg collagenase type II (lot #M6C152, 347 U/ml; Worthington Biochemical Corp., Freehold, NJ). After digestion with collagenase (20 min), the ventricles were minced and shaken in Krebs-Henseleit buffer, and the resulting cellular digest was washed, filtered, and resuspended in phosphate-free HEPES-buffered saline (HBS) containing the following (in mM): 118 NaCl, 4.8 KCl, 1.2 $MgCl_2$, 1.25 $CaCl_2$, 11.0 dextrose, 25.0 HEPES, and 5.0 pyruvate, *p*H 7.35. The solution was vigorously bubbled immediately before use with 100% O_2. Typically, $6-8 \times 10^6$ cells per rat heart were obtained using this procedure. Viability, as assessed by the percent of cells retaining a rod-like shape with no blebs or granulations, was routinely 80–90%. Myocytes were suspended in HBS (1×10^6 cells/ml) and stored in an O_2 hood until used.

Measurements of Contractility and $[Ca^{2+}]_i$

Simultaneous measurement of contraction and $[Ca^{2+}]_i$ was performed as previously described.[19] Ventricular myocytes (0.5×10^6 cells/ml) were incubated in HBS containing 2 μM fura-2/acetoxy methylester (AM) at 37°C for 20 min. Fura-2–loaded ventricular myocytes were placed in a temperature regulated (28°C) chamber (Bioptechs, Inc., Butler, PA) mounted on the stage of an Olympus IX-70 inverted fluorescence microscope (Olympus America, Lake Success, NY). This temperature was used because it minimizes loss of dye from the cells and is commonly used by other investigators.[20,21] In addition, it reduces the likelihood of spontaneous contractions or Ca^{2+} overload, which can result in contracture and cessation of contractility before completion of the protocol. The volume of the chamber was 1.5 ml. The cells were superfused continuously with HBS at a flow rate of 2 ml/min and field stimulated *via* bipolar platinum electrodes at a frequency of 0.3 Hz and for 5 ms using a Grass SD9 stimulator (Grass Instruments, West Warwick, RI). Myocytes exhibiting a rod-shaped appearance with clear striations, no membrane blebs, and a negative staircase of twitch performance on stimulation from rest were chosen for study.

Fluorescence measurements were performed on single ventricular myocytes using a dual-wavelength spectrofluorometer (Deltascan RFK6002; Photon Technology International, South Brunswick, NJ) at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. The cells also were illuminated with red light at a wavelength higher than 600 nm for simultaneous video-edge detection. An additional postspecimen dichroic mirror deflects light at wavelengths >600 nm into a CCD video camera (Phillips VC 62505T; Marshall Electronics, Culver City, CA) for measurement of myocyte shortening and relengthening. The fluorescence sampling frequency was 100 Hz, and data were collected using software from Photon Technology International (Felix). $[Ca^{2+}]_i$ was estimated by comparing the cellular fluorescence ratio with fluorescence ratios acquired using fura-2 (free acid) in buffers containing known concentrations of Ca^{2+}.

Simultaneous measurement of cell shortening was monitored using a video-edge detector (Crescent Electronics, Sandy, UT) with a 16-ms temporal resolution. The video-edge detector was calibrated using a stage micrometer so that cell lengths during shortening and relengthening could be monitored. Lab View (National Instruments, Austin, TX) was used for acquisition of data regarding cell shortening using a sampling rate of 100 Hz.

Analysis of Intracellular Ca^{2+} *Transients and Contractile Data*

Fluorescence data for $[Ca^{2+}]_i$ were imported into Labview, in which $[Ca^{2+}]_i$ and myocyte contractile responses were analyzed synchronously and simultaneously. The following parameters were calculated for each individual contraction: diastolic $[Ca^{2+}]_i$ and cell length; systolic $[Ca^{2+}]_i$ and cell length; change in $[Ca^{2+}]_i$

and twitch amplitude; time to peak (Tp) for $[Ca^{2+}]_i$ and peak shortening; and time to 50% (Tr) diastolic $[Ca^{2+}]_i$ and 50% relengthening. Parameters from 15 contractions ($[Ca^{2+}]_i$ and shortening) were averaged to obtain mean values at baseline and in response to the various interventions. Averaging the parameters over time minimizes beat-to-beat variation.

Myocyte length in response to field stimulation was measured (in micrometers) and is expressed as the change from resting cell length (twitch amplitude). Changes in twitch amplitude in response to the interventions are expressed as a percent of baseline shortening. Changes in timing were measured in milliseconds and were normalized to changes in amplitude. Changes in $[Ca^{2+}]_i$ were measured as the change in the 340/380 ratio from baseline. Changes in the 340/380 ratio in response to the interventions are expressed as a percent of the control response in the absence of any intervention.

Measurement of Intracellular pH

Intracellular *p*H was measured in myocytes using the acetoxy methylester form of 2′, 7′-bis-(2-carboxy-ethyl)-5, 6-carboxyfluorescein (BCECF/AM; Texas Fluorescence Labs, Inc., Austin, TX). Loading of BCECF/AM into ventricular myocytes was identical to the procedure described for fura-2; however, fluorescence measurements were performed using excitation wavelengths of 440 and 500 nm and an emission wavelength of 530 nm. The fluorescence sampling frequency was 10 Hz, and data were collected as described for $[Ca^{2+}]_i$. The ratio of 500 to 440 nm fluorescence values was used to estimate pH_i. At the end of the experiment, the excitation ratio from each cell was calibrated *in situ* by exposing cells to solutions of different *p*H. Each solution contained (in mM unless otherwise stated) K^+ 140, $MgCl_2$ 1.0, HEPES 4.0, EGTA 2.0, 2,3-butanedione monoxime 30, BAPTA-AM (Molecular Probes, Inc., Eugene, OR) 50 μM, and nigericin 14 μM and was titrated to different *p*H values (6.7, 6.8, 7.0, 7.2, 7.4, and 7.8) using 1.0 N KOH. A linear relationship existed between *p*H and fluorescence ratios over the *p*H range 6.5–7.8.

Purification of Sarcoplasmic Reticulum Vesicles

Freshly isolated adult rat hearts were homogenized in 5 volumes of 3-[N-morpholino]propanesulfonic acid (MOPS) buffer (10 mM, *p*H 7.4, 4°C) containing sucrose (290 mM), NaN_3 (3 mM), dithiothreitol (1 mM), pepstatin A (1 μM), leupeptin (1 μM) and phenylmethylsulfonyl fluoride (0.8 mM), *p*H 7.4, using a homogenizer (Brinkmann Polytron, Westbury, NY). The homogenate was centrifuged at 7,500*g* (20 min). The supernatant was saved and centrifuged again at 40,000*g* (60 min). The resultant pellet was suspended in three volumes of MOPS (10 mM, *p*H 6.8, 4°C) containing KCl (600 mM), NaN_3 (3 mM), dithiothreitol (1 mM), and protease inhibitors. The material was centrifuged at 140,000*g* (40 min) and the final pellet resuspended in a Ca^{2+}-free sucrose buffer and stored at −80°C until used.

Measurement of Uptake and Content of Ca^{2+} by the Sarcoplasmic Reticulum in Sarcoplasmic Reticulum Vesicles

Double-distilled tap water was deionized using a Milli-Q reagent water system (Millipore Corp., Bedford, MA) and further purified by dual ion exchange chromatography and a Ca^{2+} Sponge-S (Molecular Probes) to remove residual Ca^{2+}. A buffering system representing intracellular conditions and capable of regenerating adenosine triphosphate was used for suspending the vesicles and contained the following (in mM): HEPES 20, KCl 100, NaCl 5, $MgCl_2$ 5, and creatine phosphate 5 (*p*H 7.2, 37°C) and creatine phosphokinase (0.4 U/ml). Oxalate (10 mM) was added to act as a Ca^{2+} precipitating anion inside the vesicles to minimize leakage of Ca^{2+} and maintain the Ca^{2+} gradient across the vesicular membrane.[22] The solutions were prepared using an iterative solution mixing program (Solwin v2.0, Philadelphia, PA). Binding constants for the ionic compounds were corrected for temperature and ionic strength. $CaCl_2$ was added back to the buffer to yield a concentration of free Ca^{2+} of 1 μM (pCa 6).

Measurements of uptake and release of Ca^{2+} were examined in real time using suspensions of SR vesicles and 2 μM fura-2 free acid (Texas Fluorescence Labs) in the extravesicular compartment. Fluorescence experiments were performed using dual wavelength fluorometry in a temperature-regulated sample compartment (37°C). Microcuvettes (250 μl) were washed in EGTA (2 mM) solution to remove all Ca^{2+} and then thoroughly rinsed with Ca^{2+}-free buffer and allowed to dry. For studies on uptake of Ca^{2+}, adenosine triphosphate (1 mM) was added to the vesicular suspension to trigger the uptake of Ca^{2+} into the vesicles, which was measured as a decrease in the fluorescence signal (340/380 ratio) from the extravesicular compartment. Caffeine (20 mM) was used to release Ca^{2+} from the vesicles to examine vesicular content of Ca^{2+}. Fluorescence data were collected using Felix at a sampling frequency of 20 Hz. The rate of uptake of Ca^{2+} was measured as the

decrease in the fluorescence signal over 60 s in the presence or absence of thiopental. Addition of thiopental (30–1,000 μM) did not alter the *p*H of the suspension buffer.

Experimental Protocols

Protocol 1: Dose-dependent Effects of Thiopental on $[Ca^{2+}]_i$ and Myocyte Shortening. Changes in myocyte shortening and $[Ca^{2+}]_i$ during exposure to thiopental were determined. Baseline measurements were collected from individual myocytes for 1.5 min in the absence of any intervention. Myocytes were exposed to four concentrations of thiopental (30, 100, 300, and 1,000 μM) by exchanging the buffer in the dish with new buffer containing thiopental at the desired concentration. Data were acquired for 1.5 min after a 5-min equilibration period in the presence of the anesthetic agent.

Protocol 2: Effect of Thiopental on Ca^{2+} Stores in Sarcoplasmic Reticulum. To determine whether thiopental alters release of Ca^{2+} from intracellular Ca^{2+} stores, we measured caffeine-induced release of Ca^{2+} in the presence or absence of the anesthetic agent. Baseline values for $[Ca^{2+}]_i$ were measured in individual, field-stimulated myocytes for 1.5 min. Thiopental (100 and 1,000 μM) was then added to the superfusion buffer and allowed to equilibrate for 5 min. Field stimulation of the myocyte was discontinued, and caffeine (20 mM) was applied to the cell 15 s later. Peak $[Ca^{2+}]_i$ induced by caffeine was compared with peak $[Ca^{2+}]_i$ before addition of thiopental and is reported as a percent of the control amplitude.

Protocol 3: Effect of Thiopental on Myofilament Ca^{2+} Sensitivity. To determine whether thiopental alters myofilament Ca^{2+} sensitivity, we examined the dose–response curve to the extracellular concentration of Ca^{2+} ($[Ca^{2+}]_o$) in the presence or absence of thiopental. Baseline parameters were collected from individual myocytes for 1.5 min. Dose–response curves for $[Ca^{2+}]_o$ were performed by exchanging the buffer in the dish with a new buffer containing the desired $[Ca^{2+}]_o$. Data were acquired for 1.5 min after establishment of a new steady state. Dose–response curves for $[Ca^{2+}]_o$ were then performed in the presence of thiopental (100 μM). This concentration was chosen because we wanted to assess myofilament Ca^{2+} sensitivity in the absence of any significant cardiac depression. Cells were allowed to stabilize for 5 min after addition of each intervention. The data for cell shortening were plotted against $[Ca^{2+}]_o$ and peak $[Ca^{2+}]_i$. The relative contribution of the Na^+–H^+ exchanger in mediating changes in myofilament Ca^{2+} sensitivity was assessed using the same protocol, except the cells were pretreated (5 min) with 1 μM 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA) before addition of thiopental.

Protocol 4: Effect of Thiopental on Intracellular *p*H. To determine the effects of thiopental on pH_i, separate experiments were performed in myocytes loaded with BCECF/AM. Myocytes were first perfused with the same HBS used for baseline superfusion to estimate the effects of Cl^-/HCO_3^- exchange and Na^+/HCO_3^- exchange on pH_i. The superfusate was then switched to HBS containing 30 or 100 μM thiopental for 15 min. To further assess the contribution of Na^+–H^+ exchange to thiopental-induced alkalinization, additional BCECF/AM-loaded myocytes were perfused with EIPA (1 μM). As described earlier, all experiments were performed using HBS with an extracellular *p*H of 7.4. The pH_i was measured at baseline and after 5 min of exposure to 1 μM EIPA, followed by 15 min of exposure to 0, 30, or 100 μM thiopental in the presence of EIPA.

We have verified that thiopental has no effect on the fura-2 or BCECF signal at the concentrations tested. This was confirmed in separate experiments using fura-2 or BCECF free acid in HBS and examining whether thiopental altered the 340/380 or 500/440 ratios. In addition, thiopental had no effect on the *p*H of the bath solution within the range 30–1,000 μM. EIPA alone did not cause any changes in baseline pH_i; however, EIPA interfered with fura-2 fluorescence measurements, so it was not possible to assess its effect on peak $[Ca^{2+}]_i$ in response to increasing $[Ca^{2+}]_o$.

Statistical Analysis and Data Presentation

Each experimental protocol was performed on multiple myocytes from the same heart and repeated in at least four hearts. Results obtained from myocytes in each heart were averaged so that all hearts were weighted equally. The dose-dependent effects of thiopental on myocyte shortening and $[Ca^{2+}]_i$ were assessed using one-way analysis of variance with repeated measures and the Bonferroni/Dunn *post hoc* test or Student's *t* test when appropriate. Comparisons between groups were made by two-way analysis of variance. Results are expressed as means ± SEM. Differences were considered statistically significant at $P < 0.05$.

Materials

Sodium thiopental, caffeine, EIPA, and nigericin were purchased from Sigma Chemical Co. (St. Louis, MO). Thiopental was dissolved fresh each day before use.

Results

Baseline Parameters for Myocyte Shortening and $[Ca^{2+}]_i$

One hundred thirty-two cells were used for the study. Baseline $[Ca^{2+}]_i$ was 85 ± 10 nM, and the diastolic cell length was 127 ± 5 μm. Peak $[Ca^{2+}]_i$ was 355 ± 22 nM. Twitch amplitude was 10% (12.8 ± 0.8 μm) of the baseline diastolic resting cell length. Time to peak (Tp) for $[Ca^{2+}]_i$ and shortening were 139 ± 6 and 173 ± 7 ms, respectively. Time to 50% recovery (Tr) for $[Ca^{2+}]_i$ and shortening were 296 ± 6 and 290 ± 6 ms, respectively.

Effect of Thiopental on Myocyte Shortening and $[Ca^{2+}]_i$

Figure 1A demonstrates that addition of thiopental to a single, field-stimulated ventricular myocyte resulted in dose-dependent inhibition of myocyte shortening and a concomitant decrease in peak $[Ca^{2+}]_i$. The myocardial depressant effects of thiopental were reversed completely after washout. Individual contractions and $[Ca^{2+}]_i$ transients are illustrated in figure 1B. Thiopental had no effect on resting $[Ca^{2+}]_i$ or cell length. The summarized data are shown in figure 2. Thiopental caused dose-dependent decreases in myocyte shortening and peak $[Ca^{2+}]_i$. At the highest concentration tested, thiopental (1,000 μM) decreased myocyte shortening and peak $[Ca^{2+}]_i$ to 37 ± 3% and 58 ± 7% of control, respectively.

Thiopental prolonged Tp for $[Ca^{2+}]_i$ at all concentrations tested (fig. 3). The lowest dose of thiopental (30 μM) decreased Tp for shortening, whereas 1,000 μM thiopental increased Tp for shortening. Thiopental increased Tr for $[Ca^{2+}]_i$ and shortening at the highest concentration tested (1,000 μM).

Effect of Thiopental on Uptake and Content of Ca^{2+} *in Isolated Sarcoplasmic Reticulum Vesicles*

We assessed the extent to which thiopental altered the initial rate of uptake and content of Ca^{2+} into isolated SR vesicles. The rate of uptake of Ca^{2+} by the vesicles was measured in real time as a decrease in the 340/380 ratio from the extravesicular compartment. Caffeine (20 mM) was used to release Ca^{2+} from the vesicles. Figure 4A demonstrates that addition of thiopental caused dose-dependent inhibition in the rate of uptake of Ca^{2+} into the SR vesicles. The total amount of Ca^{2+} released from the vesicles in response to caffeine, however, was unaltered (98 ± 3% of control) by thiopental (fig. 4A). The summarized data for the effects of thiopental on the rate of uptake of Ca^{2+} into SR vesicles are shown in figure 4B. Thiopental (30 μM) decreased the rate of uptake of Ca^{2+} into the vesicles by 28 ± 4%. Higher concentrations of thiopental (300 μM) decreased the rate of uptake into the SR vesicles by 60 ± 6%.

Effect of Thiopental on Caffeine-induced Release of Ca^{2+} *from the SR in Myocytes*

We also assessed the extent to which thiopental altered the amount of Ca^{2+} released from the SR in response to caffeine (20 mM) in intact myocytes. In control myocytes, rapid exposure to caffeine caused a transient increase in $[Ca^{2+}]_i$, which was 99 ± 5% of peak $[Ca^{2+}]_i$ induced by field stimulation. Thiopental (100 and 1,000 μM) did not alter the amplitude of the caffeine-releasable pool of Ca^{2+} compared with that observed with the control response to caffeine (fig. 5).

Effect of Thiopental on the Dose-Response Curve to $[Ca^{2+}]_o$

The effect of $[Ca^{2+}]_o$ on myocyte shortening and peak $[Ca^{2+}]_i$ was examined in the presence and absence of thiopental. Increasing $[Ca^{2+}]_o$ from 1 to 4 mM (control, without thiopental) resulted in a dose-dependent increase in shortening and a concomitant increase in peak $[Ca^{2+}]_i$ (fig. 6). Thiopental (100 μM) caused an upward shift in the dose-response curve to $[Ca^{2+}]_o$ for shortening, with no concomitant effect on peak $[Ca^{2+}]_i$ (fig. 6). In addition, thiopental caused a significant leftward shift in the relationship between peak $[Ca^{2+}]_i$ and cell shortening (fig. 7).

Effect of Thiopental on Intracellular pH

The effects of thiopental on pH_i in cardiac myocytes are shown in figure 8. Baseline pH_i was 7.08 ± 0.01. Thiopental (30 and 100 μM) caused a significant increase in pH_i after only 1 min, and this increase lasted for at least 15 min. To assess the potential contribution of Na^+-H^+ exchange on this thiopental-induced alkalinization, additional cells were exposed to 1 μM EIPA for 5 min (to inhibit Na^+-H^+ exchange) and then exposed to 100 μM thiopental for 15 min during continued exposure to EIPA. In the presence of EIPA, 100 μM thiopental failed to cause intracellular alkalinization in myocytes (fig. 8).

Effect of EIPA on the Thiopental-induced Upward Shift in the Dose-Response Curve to $[Ca^{2+}]_o$

We assessed the contribution of the Na^+-H^+ exchanger in mediating the thiopental-induced upward

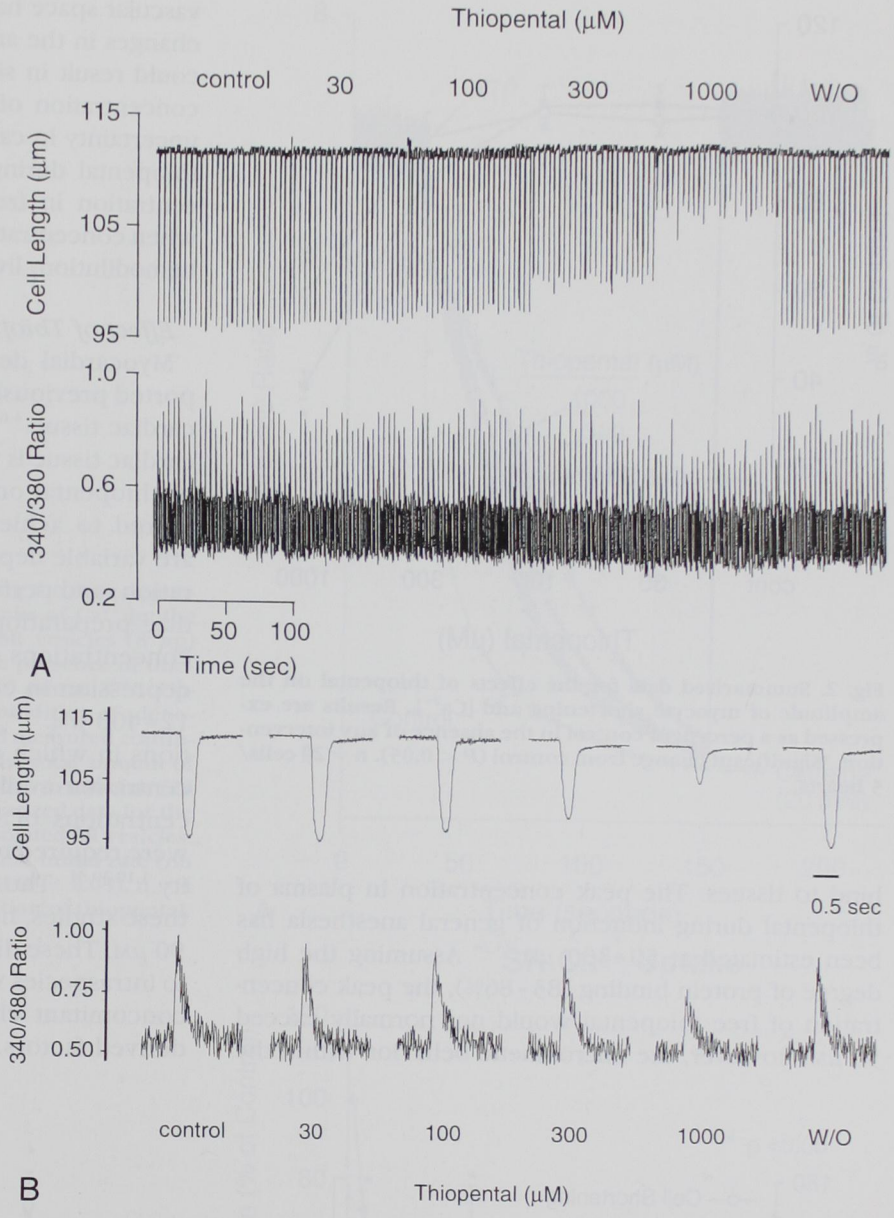

Fig. 1. (*A*) Representative trace demonstrating the dose-dependent inhibitory effect of thiopental on myocyte shortening (*top*) and intracellular concentration of free Ca^{2+} ($[Ca^{2+}]_i$) in a single ventricular myocyte. Thiopental was added at the concentrations depicted in the figure. $[Ca^{2+}]_i$ was measured as the 340/380 fluorescence ratio. (*B*) Exploded view of individual contractions and $[Ca^{2+}]_i$ transients from figure 1A. W/O = washout.

shift in the dose-response curve to $[Ca^{2+}]_o$ for myocyte shortening. The results are summarized in figure 9. In the presence of EIPA (1 μM), thiopental (100 μM) failed to cause an upward shift in the dose-response curve to $[Ca^{2+}]_o$.

Discussion

This is the first study to simultaneously assess the effects of thiopental on cardiomyocyte contractility and $[Ca^{2+}]_i$ at the cellular level and to examine the effects of thiopental on pH_i. Thiopental decreased shortening and peak $[Ca^{2+}]_i$ but increased pH_i in a dose-dependent fashion; however, the concentrations of thiopental required to achieve cardiac depression in this model are higher than those encountered clinically.

Binding of Thiopental to Plasma Proteins

All anesthetic agents bind to plasma proteins, reducing the concentration of anesthetic agent available to

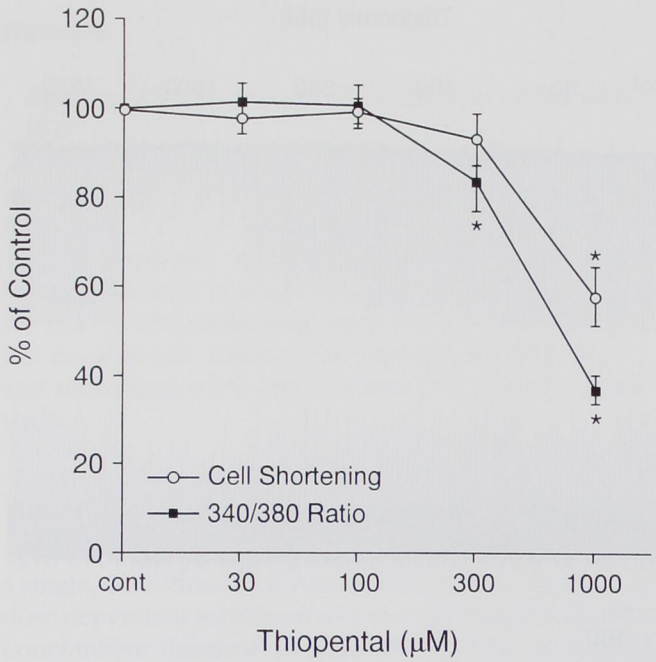

Fig. 2. Summarized data for the effects of thiopental on the amplitude of myocyte shortening and $[Ca^{2+}]_i$. Results are expressed as a percent of control in the absence of any intervention. *Significant change from control ($P < 0.05$). n = 20 cells/5 hearts.

bind to tissues. The peak concentration in plasma of thiopental during induction of general anesthesia has been estimated at 50–300 μM.[23,24] Assuming the high degree of protein binding (83–86%), the peak concentration of free thiopental would not normally exceed 50 μM; however, the microkinetic behavior within the vascular space has not been defined. In addition, small changes in the amount or binding capacity of proteins could result in significant increases in the free plasma concentration of anesthetic agents. Not only is there uncertainty in calculating the *in vivo* concentration of thiopental during normal circumstances but the concentration in free plasma would certainly be higher when concentration of protein in serum is reduced (*e.g.*, hemodilution, liver disease, hypoproteinemia).

Effect of Thiopental on Contractility and $[Ca^{2+}]_i$

Myocardial depression by thiopental has been reported previously in humans,[1,2] animals,[25] and isolated cardiac tissue.[4,6–11,16,26,27] Although the use of isolated cardiac tissue is useful for examining the direct effects of thiopental on contractility, the concentrations required to achieve significant contractile depression are variable depending on the species, cardiac preparation, and perfusion medium. In buffer-perfused cardiac preparations from several different species, the concentrations of thiopental required to cause a 50% depression in contractility are variable, ranging from 12–400 μM.[4–6,8,11,13,26,28,29] In blood-perfused preparations in which protein binding reduces the free concentration available to interact with the tissue, concentrations of thiopental ranging from 70–568 μM were required to cause a 50% depression in contractility.[1,10,30,31] The concentration of free thiopental in these studies, however, was likely in the range of 20–90 μM. These differential results are likely attributable to intraspecies variation; experimental preparation; or concomitant changes in neural, humoral, or locally derived factors.

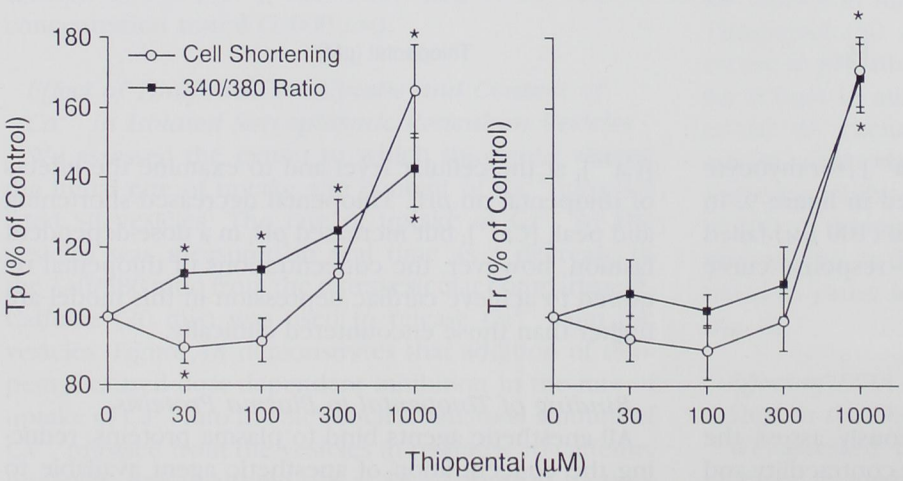

Fig. 3. Summarized data for the effects of thiopental on time to peak (Tp) and time to 50% recovery (Tr) for myocyte shortening and $[Ca^{2+}]_i$. Changes in timing were measured in milliseconds and were normalized to changes in peak amplitude. *Significant change from control ($P < 0.05$). n = 20 cells/5 hearts.

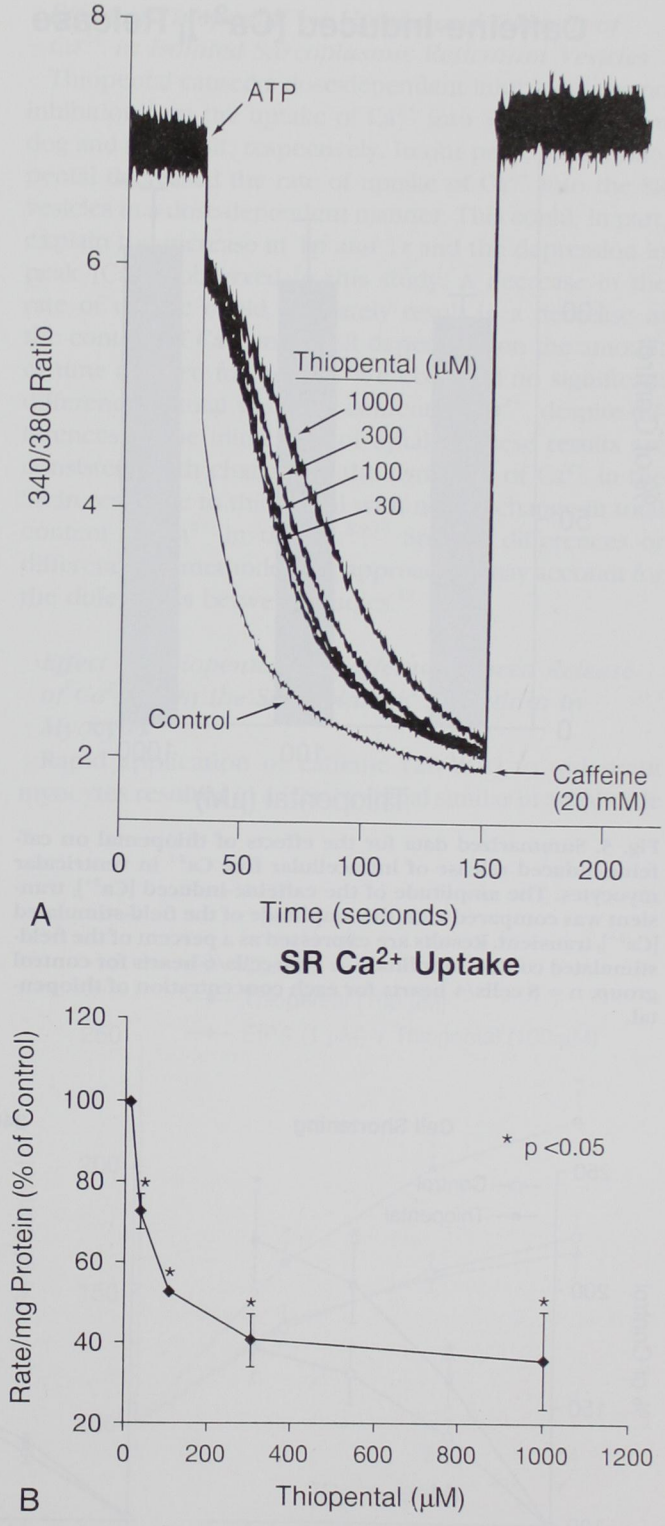

Fig. 4. (*A*) Representative trace demonstrating the dose-dependent inhibitory effect of thiopental on uptake of Ca^{2+} by the sarcoplasmic reticulum (SR). Aliquots of SR vesicles (8 μg) were preincubated in buffer alone or in the presence of thiopental at the concentrations indicated for 10 min at 37°C. Uptake of Ca^{2+} into SR vesicles was triggered by addition of adenosine triphosphate to the cuvette (*arrow*) and measured continuously as a decrease in the 340/380 ratio. The total amount of Ca^{2+} released from the SR vesicles in response to caffeine (20 mM) was unaltered by thiopental. (*B*) Summarized data for the effects of thiopental on uptake of Ca^{2+} in isolated SR vesicles. Results are expressed as a percent of maximal initial rate (no treatment). *Significant change from control ($P < 0.05$). n = 4 determinations/6 hearts for each concentration of thiopental.

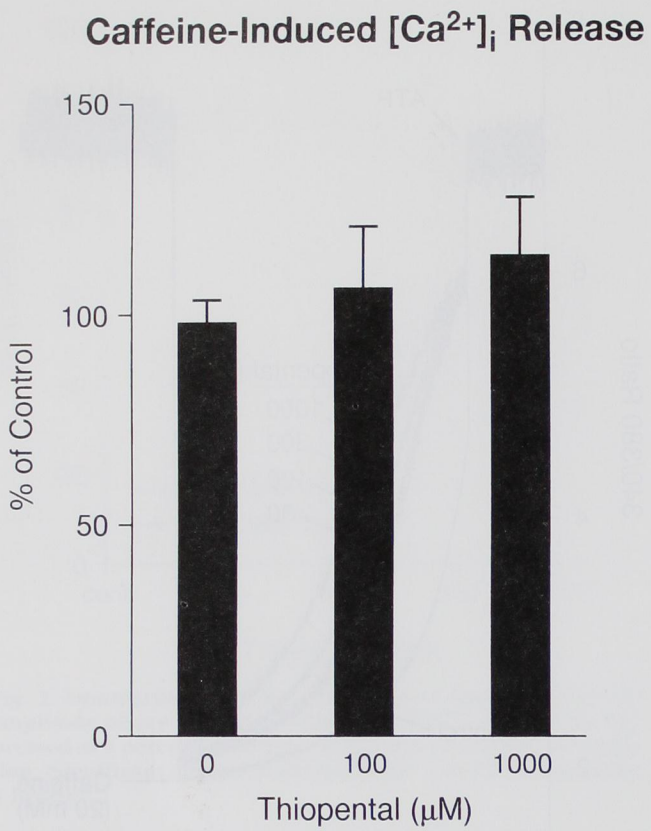

Fig. 5. Summarized data for the effects of thiopental on caffeine-induced release of intracellular free Ca^{2+} in ventricular myocytes. The amplitude of the caffeine-induced $[Ca^{2+}]_i$ transient was compared with the amplitude of the field-stimulated $[Ca^{2+}]_i$ transient. Results are expressed as a percent of the field-stimulated control amplitude. n = 16 cells/6 hearts for control group; n = 8 cells/4 hearts for each concentration of thiopental.

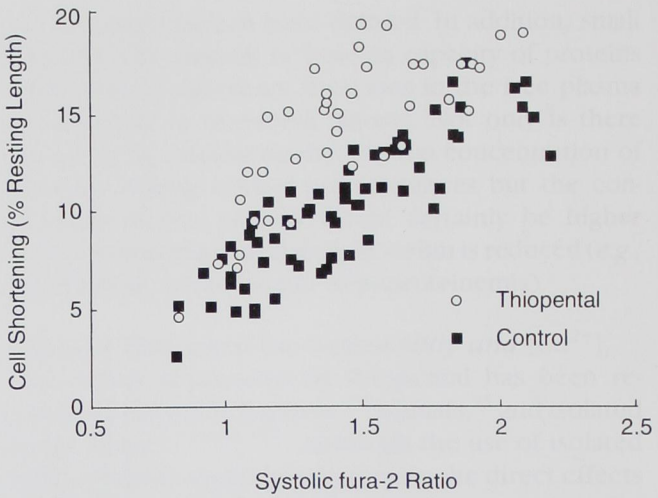

Fig. 7. The effect of thiopental on myofilament Ca^{2+} sensitivity was assessed by plotting twitch shortening (percent of resting cell length) as a function of peak $[Ca^{2+}]_i$ (systolic fura-2 ratio) in the absence or presence of thiopental (100 μM). $[Ca^{2+}]_i$ was varied by increasing $[Ca^{2+}]_o$. The shortening-$[Ca^{2+}]_i$ relation obtained during control conditions (*filled squares*) was shifted to the left by thiopental (*open circles*).

In our study, the negative inotropic effect of thiopental was reversible on washout, indicating that the effect was not toxic. Inhibition of transsarcolemmal entry of Ca^{2+} is thought to be one possible explanation. Thiopental attenuated peak $[Ca^{2+}]_i$ in ferret papillary muscle[6] and inhibited the L-type Ca^{2+} current in rat[14] and guinea pig[12] ventricular myocytes. In this study, the inhibitory effects of thiopental were correlated with a reduction in peak $[Ca^{2+}]_i$, which is likely mediated, at least in part, *via* inhibition of sarcolemmal L-type Ca^{2+} currents.

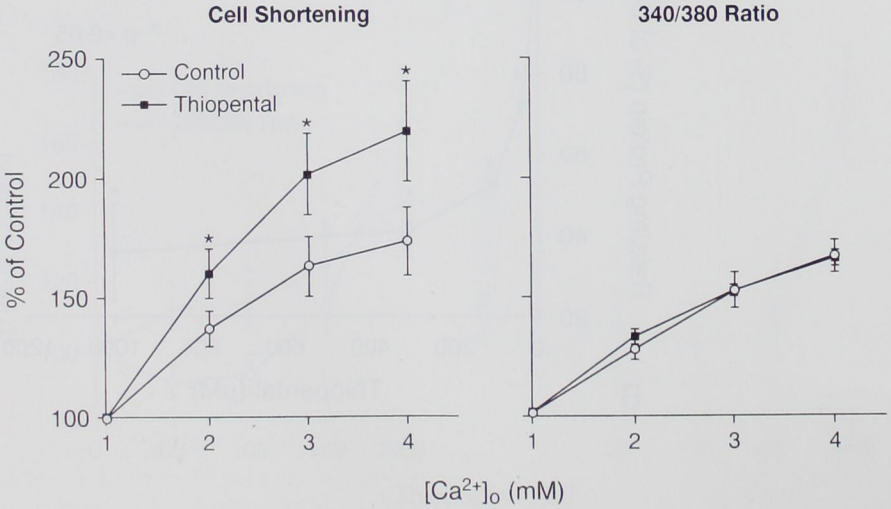

Fig. 6. Summarized data for the effects of thiopental on myocyte shortening and $[Ca^{2+}]_i$ transients in response to increasing extracellular concentration of Ca^{2+} ($[Ca^{2+}]_o$). Baseline values: cell length = 122 ± 4 μm; twitch amplitude = 11 ± 1 μm; shortening = 9 ± 1%; diastolic $[Ca^{2+}]_i$ = 80 ± 12 nM; peak $[Ca^{2+}]_i$ = 302 ± 30 nM. *Significant change from control ($P < 0.05$). n = 24 cells/6 hearts for control group; n = 20 cells/5 hearts for thiopental group.

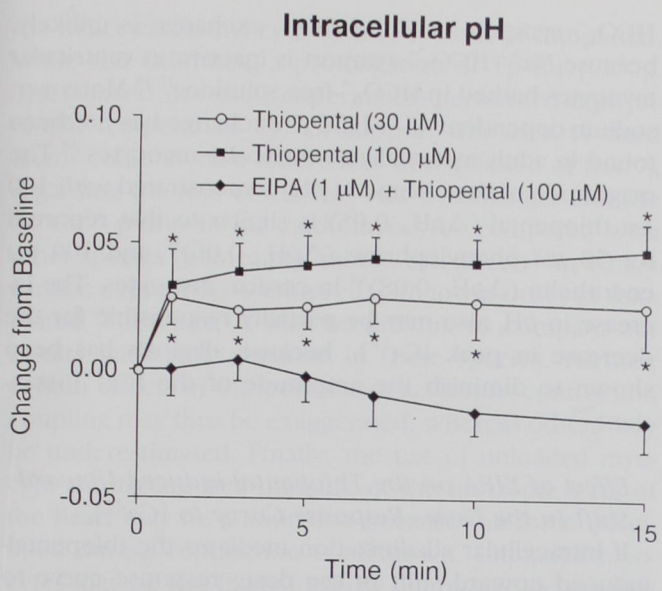

Fig. 8. Time course effects of thiopental alone and after pretreatment with ethylisopropyl amiloride (EIPA) on intracellular *p*H (pH_i) in rat ventricular myocytes. Values are normalized relative to baseline for each myocyte. *Significant change from baseline ($P < 0.05$). n = 20 cells/5 hearts for each concentration of thiopental.

Effect of Thiopental on Time to Peak and Time to 50% Recovery for Myocyte Shortening and $[Ca^{2+}]_i$

Changes in the timing parameters would suggest alterations in ion channel activity, SR cycling of Ca^{2+}, or myofilament Ca^{2+} sensitivity. Several reports indicate that thiopental alters the dynamics of Ca^{2+} in the SR.[8,11] Our results are consistent with a decrease in the rate of release of Ca^{2+} from the SR[9,11] or inhibition of the uptake of Ca^{2+} by the SR.[11,15,32] Alternatively, inhibition of K^+ currents by thiopental[12,16] could prolong Tp for $[Ca^{2+}]_i$. The decrease in Tp for shortening observed with thiopental (30 μM), in the presence of an increase in Tp for $[Ca^{2+}]_i$, suggests that thiopental may increase myofilament Ca^{2+} sensitivity. This may be attributable to the intracellular alkalinization and enhanced affinity of troponin C for Ca^{2+}. Prolongation of Tp and Tr for both $[Ca^{2+}]_i$ and shortening were observed at the highest concentration tested, along with a decrease in peak $[Ca^{2+}]_i$, which is consistent with direct inhibition of uptake of Ca^{2+} by the SR. Alternative explanations include an indirect effect on uptake of Ca^{2+} by the SR because of a decrease in peak $[Ca^{2+}]_i$.[20] An increase in the affinity of troponin C for Ca^{2+} could also prolong Tr. Therefore, thiopental likely has multiple sites of action at the cellular level.

Effect of Thiopental on Uptake and Content of Ca^{2+} in Isolated Sarcoplasmic Reticulum Vesicles

Thiopental caused a dose-dependent inhibition[15] or no inhibition[17] on the uptake of Ca^{2+} into SR vesicles from dog and rabbit SR, respectively. In our preparation, thiopental decreased the rate of uptake of Ca^{2+} into the SR vesicles in a dose-dependent manner. This could, in part, explain the increase in Tp and Tr and the depression in peak $[Ca^{2+}]_i$ observed in this study. A decrease in the rate of uptake could ultimately result in a decrease in the content of Ca^{2+} in the SR depending on the amount of time allowed for uptake. We observed no significant difference in total vesicular content of Ca^{2+}, despite differences in the initial rate of uptake. These results are consistent with changes in the dynamics of Ca^{2+} in the SR in response to thiopental with no net change in total content of Ca^{2+} in the SR.[8,9,15] Species differences or differences in methodologic approaches may account for the differences between studies.[17]

Effect of Thiopental on Caffeine-induced Release of Ca^{2+} from the Sarcoplasmic Reticulum in Myocytes

Rapid application of caffeine (20 mM) to quiescent myocytes resulted in a $[Ca^{2+}]_i$ signal similar in amplitude

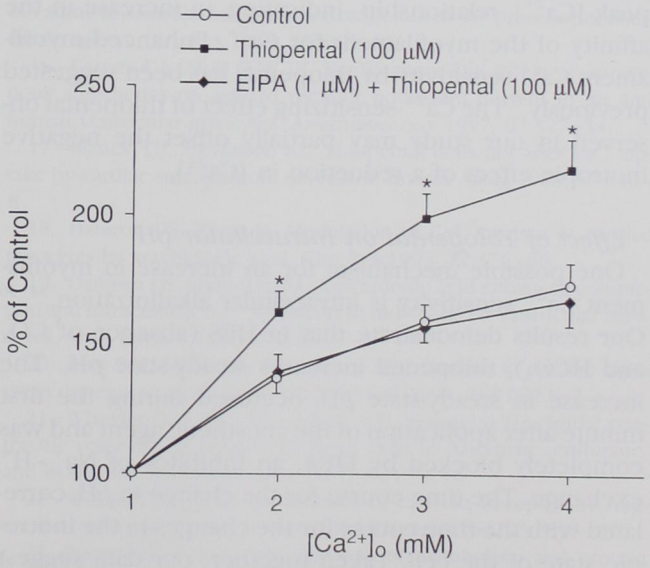

Fig. 9. Summarized cell shortening data for the effect of EIPA on the thiopental-induced upward shift in the dose–response curve to $[Ca^{2+}]_o$. *Significant change from control ($P < 0.05$). n = 12 cells/3 hearts for each group.

to that observed with field stimulation. Thiopental, however, did not alter the amplitude of the caffeine-induced $[Ca^{2+}]_i$ transient even at the highest concentration tested. These results are consistent with our data obtained in the isolated SR vesicles indicating no change in content of Ca^{2+} in the SR after treatment with thiopental. It has been proposed that thiopental may inhibit release of Ca^{2+} by the SR without decreasing the content of Ca^{2+} in the SR.[9] Our results indicate that thiopental does not exert a negative inotropic effect by altering the content of Ca^{2+} in the SR.

Effect of Thiopental on the Dose-Response Curve to $[Ca^{2+}]_o$

Because alterations in myofilament Ca^{2+} sensitivity can alter contractility, we examined whether thiopental altered dose-response curves to $[Ca^{2+}]_o$.[33] This protocol allowed for paired comparisons of $[Ca^{2+}]_i$ and contractile amplitude in the same cell in the presence or absence of thiopental, over a range of more than one $[Ca^{2+}]_o$. Thiopental caused an upward shift in the dose-response curve to $[Ca^{2+}]_o$ for shortening, with no concomitant effect on $[Ca^{2+}]_i$. Because an increase in $[Ca^{2+}]_o$ results in an increase in $[Ca^{2+}]_i$, these data suggest that the negative inotropic effect of thiopental is diminished when $[Ca^{2+}]_i$ is increased. Therefore, thiopental may increase the maximal response of the myofilament to Ca^{2+} as $[Ca^{2+}]_i$ increases. Thiopental also caused a leftward shift in the cell shortening *versus* peak $[Ca^{2+}]_i$ relationship, indicating an increase in the affinity of the myofilament for Ca^{2+}. Enhanced myofilament Ca^{2+} sensitivity by thiopental has been suggested previously.[6] The Ca^{2+}-sensitizing effect of thiopental observed in our study may partially offset the negative inotropic effect of a reduction in $[Ca^{2+}]_i$.

Effect of Thiopental on Intracellular pH

One possible mechanism for an increase in myofilament Ca^{2+} sensitivity is intracellular alkalinization.[34-36] Our results demonstrate that in HBS (absence of CO_2 and HCO_3), thiopental increases steady-state pH_i. The increase in steady-state pH_i occurred during the first minute after application of the anesthetic agent and was completely blocked by EIPA, an inhibitor of Na^+-H^+ exchange. The time course for the change in pH_i correlated with the time course for the changes in the inotropic state of the cell. Taken together, our data suggest that the thiopental-induced intracellular alkalinization is mediated by stimulation of Na^+-H^+ exchange. Possible involvement of thiopental-induced changes in Na^+-HCO_3^- symport or Cl^--HCO_3^- exchange is unlikely, because Na^+-HCO_3^- symport is inactive in ventricular myocytes bathed in HCO_3^--free solution.[37,38] Moreover, sodium-dependent Cl^--HCO_3^- exchange has not been found in adult mammalian ventricular myocytes.[38] The magnitude of the change in pH_i demonstrated with 100 μM thiopental (ΔpH_i, 0.05) is similar to that reported for 50 μM phenylephrine (ΔpH_i, 0.06)[36] and 100 pM endothelin (ΔpH_i, 0.08)[34] in cardiac myocytes. The increase in pH_i also may be partially responsible for the decrease in peak $[Ca^{2+}]_i$, because alkalosis has been shown to diminish the amplitude of the $[Ca^{2+}]_i$ transient.[39]

Effect of EIPA on the Thiopental-induced Upward Shift in the Dose-Response Curve to $[Ca^{2+}]_o$

If intracellular alkalinization mediates the thiopental-induced upward shift in the dose-response curve to $[Ca^{2+}]_o$ for shortening, then this effect should be reversed by inhibiting the Na^+-H^+ exchanger with EIPA. Our results indicate that inhibition of Na^+-H^+ exchange activity with EIPA prevents the thiopental-induced increase in myofilament Ca^{2+} sensitivity. These data suggest that the negative inotropic effect of thiopental is diminished when $[Ca^{2+}]_o$ is increased, and this effect is abolished by EIPA. This result is similar to other reports in which inhibition of Na^+-H^+ exchange with amiloride attenuates endothelin-[34] and phenylephrine-[36] enhanced myofilament Ca^{2+} sensitivity in rat ventricular myocytes.

Limitations

There are several factors that could explain why high doses of thiopental were required to achieve a significant decrease in myocyte shortening. First, our results clearly demonstrate that thiopental has multiple actions on regulation of $[Ca^{2+}]_i$. It is conceivable that the effects of thiopental on uptake of Ca^{2+} by the SR and Tp for $[Ca^{2+}]_i$ could counteract the direct cardiodepressant action resulting from reduced Ca^{2+} entry. Moreover, the negative inotropic effect of thiopental is likely masked by the concomitant increase in myofilament Ca^{2+} sensitivity. Second, it is possible that the temperature used in this study (28°C) is "cardioprotective" and could contribute to the requirement for higher concentrations of thiopental compared with physiologic temperatures (37°C). In addition, enzymatic activity (*e.g.*, SR Ca^{2+} adenosine triphosphatase) is likely to be reduced to a greater extent than ion transport processes. This could mask thiopental-induced alterations in Tr for $[Ca^{2+}]_i$ in

the intact cardiomyocyte with low doses of thiopental, while still exhibiting a prolongation of Tp for $[Ca^{2+}]_i$. The isolated SR vesicle experiments performed at physiologic temperature (37°C) support this idea. A third factor could involve differences in regulation of $[Ca^{2+}]_i$ regarding the role of the Na^+-Ca^{2+} exchanger and the SR Ca^{2+} pump in rat cardiomyocytes compared with other species (guinea pig, rabbit, cat, ferret)[40] in which cardiac depression is achieved with lower doses of thiopental. Therefore, it may be difficult to compare our results with data obtained in these species. Further, certain effects of thiopental on excitation-contraction coupling may thus be exaggerated, whereas others may be underestimated. Finally, the use of unloaded myocyte shortening as a measure of the inotropic state of the heart may be a limitation because the force developed during contraction is unknown. Unloaded myocytes may be less likely to show contractile depression in response to anesthetic agents. Despite these limitations, there is remarkable consistency between the qualitative results obtained from unloaded cells and multicellular preparations, although quantitative differences (*e.g.*, shifts in dose dependence) may exist.

Summary

The inhibitory effect of thiopental on myocyte shortening involves a decrease in the availability of $[Ca^{2+}]_i$, which may be partially counteracted by a concomitant increase in myofilament Ca^{2+} sensitivity. This latter effect is likely attributable to intracellular alkalinization *via* activation of Na^+-H^+ exchange. Thiopental altered Tp and Tr for $[Ca^{2+}]_i$ and shortening. These changes in timing are likely attributable to alterations in myofilament Ca^{2+} sensitivity or uptake of Ca^{2+} by the SR.

The authors thank Beth Summers and Cindy Shumaker for excellent technical support and Ronnie Sanders for outstanding work in preparing the manuscript.

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