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Bupivacaine Attenuates Contractility by Decreasing Sensitivity of Myofilaments to Ca^{2+} in Rat Ventricular Muscle

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Background: **Bupivacaine exhibits a cardiodepressant effect, the molecular mechanism(s) of which have yet to be fully understood. Bupivacaine may directly act on contractile proteins and thereby decrease myofibrillar Ca^{2+} sensitivity.**

Methods: **Rat ventricular muscle was used. First, the effect of bupivacaine was examined on tetanic contractions in isolated intact myocytes. Next, Triton X-100–treated ventricular trabeculae were used to investigate the effect of bupivacaine on the pCa (= −log [Ca^{2+}])–tension relation as well as on maximal Ca^{2+}-activated tension. Furthermore, to test whether bupivacaine inhibits the pathway downstream from Ca^{2+} binding to troponin C, tension was elicited in the skinned preparations by lowering the Mg-adenosine triphosphate (MgATP) concentration in the absence of Ca^{2+}. The effect of bupivacaine on the pMgATP (= −log [MgATP])–tension relation was examined.**

Results: **In myocytes, 3 μM bupivacaine significantly ($P < 0.01$) increased intracellular Ca^{2+} concentration required for 5% cell shortening from the resting cell length. In skinned preparations, bupivacaine shifted the pCa–tension relation to the lower pCa side; the midpoint of the pCa curve (pCa_{50}) was significantly ($P < 0.05$) changed by 10 and 100 μM bupivacaine. A highly correlated linear relation ($R = 0.81$; $P < 0.0005$) was present between pCa_{50} and maximal Ca^{2+}-activated tension. Bupivacaine (10 and 100 μM) significantly ($P < 0.05$) shifted the midpoint of the pMgATP–tension relation to the higher pMgATP side.**

Conclusions: **Bupivacaine decreases myofibrillar Ca^{2+} sensitivity in ventricular muscle, and this is coupled with the compound's inhibitory effect on the pathway beyond Ca^{2+} binding to troponin C, possibly on the actomyosin interaction. The current results may partly explain the overall cardiodepressant effect of bupivacaine *in vivo*.**

BUPIVACAINE is one of the most commonly used long-lasting local anesthetics in the clinical setting. It has been reported that high doses of bupivacaine markedly suppress cardiac contractile performance[1–3] and induce arrhythmia,[4–6] which may cause cardiac arrest in humans.[7] The effects of bupivacaine on cardiac muscle contraction have been extensively studied thus far. Accumulating evidence suggests that bupivacaine acts in a number of specific ways in cardiac muscle, including block of sodium,[8] calcium,[9,10] and potassium[11] channels on the sarcolemma, interference with the Ca^{2+}-induced Ca^{2+}-release mechanism from the sarcoplasmic reticulum,[12,13] collapse of the mitochondrial transmembrane potential,[14] as well as inhibition of electron transport for oxidative phosphorylation.[15] These effects may synergistically contribute to the overall cardiodepressant effect of bupivacaine. It is also possible, considering the recent findings for halothane and isoflurane,[16,17] that bupivacaine directly acts on contractile proteins and, subsequently, decreases the Ca^{2+}sensitivity of cardiac myofilaments.

It has been reported that inhalation anesthetics, such as halothane and isoflurane,[16,17] as well as opioids, such as fentanyl and morphine,[18] decrease myofibrillar Ca^{2+} sensitivity in cardiac muscle. Recently, Tavernier *et al.*[16] demonstrated that clinically relevant doses of halothane or isoflurane decrease Ca^{2+} sensitivity and suppress maximal Ca^{2+}-activated tension production in human skinned cardiac muscle. This finding provides strong evidence that a decrease in myofibrillar Ca^{2+} sensitivity, at least in part, underlies the cardiodepressant effect of halothane or isoflurane encountered in the clinical setting.

In the current study, to elucidate whether bupivacaine suppresses the contractile properties of cardiac muscle at the myofilament level, we used two experimental systems using rat ventricular muscle. First, Ca^{2+} sensitivity was quantitatively estimated by simultaneously measuring the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and cell shortening in tetanized isolated myocytes. Second, isometric tension, either Ca^{2+}-dependent or Ca^{2+}-independent, was measured in skinned muscle preparations from which the membrane system had been chemically disrupted by the treatment with 1% (vol/vol) polyethylene glycol mono-p-isooctylphenyl ether (Triton X-100; Nacalai Tesque, Kyoto, Japan) for 60 min. Our results show that bupivacaine suppresses cardiac contractility in both intact and skinned muscle preparations.

Materials and Methods

All experiments conducted in the current study conform with *The Guiding Principles for the Care and Use of Animals* approved by the Council of the Physiologic Society of Japan.

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Preparation of Intact Cardiomyocytes

Myocytes were prepared according to the previously reported procedure.[19,20] Briefly, the hearts were quickly removed from male Wistar rats (weight, 250–350 g), anesthetized with sodium pentobarbital (50 mg/kg administered intraperitoneally), and then perfused with Ca^{2+}-free HEPES–Tyrode solution and 0.04 mg/ml protease (Type XIV, Sigma, St. Louis, MO) for 3–4 min using a Langendorff column. Myocytes were isolated using an enzymatic dispersion technique, and the enzymes were washed out with the HEPES–Tyrode solution containing 0.2 mM Ca^{2+}. The isolated myocytes were stored in the HEPES–Tyrode solution containing 1 mM Ca^{2+} at 4°C before use for up to 8 h.

Measurement of Intracellular Ca^{2+} Concentration and Cell Length

Isolated myocytes were bathed in the 1-mM Ca^{2+}–HEPES–Tyrode solution containing 4 μM fura-2-AM (Molecular Probes, Eugene, OR) for 10 min at 22°C. The cells were then centrifuged for 40 s at 500 rpm to remove surplus fura-2-AM and to stop further loading of fura-2 into the cells. The fura-2–loaded myocytes were resuspended in the 1 mM Ca^{2+}–HEPES–Tyrode solution and placed in a chamber mounted on the stage of an inverted microscope (Diaphot; Nikon, Tokyo, Japan). All experiments were performed in the presence of 1 mM Ca^{2+}.[20] Excitation of the dye and detection of the emitted fluorescence from the preparation were essentially the same as described previously.[19,20] Rod-shaped myocytes were excited alternately at 340 and 380 nm at 400 Hz using an epifluorescence system (CAM-230; JASCO, Tokyo, Japan). The resultant fluorescence signals were passed through a 500 ± 20 nm bandpass filter before detecting with a photomultiplier tube (R268; Hamamatsu Photonics, Hamamatsu, Japan). After each experiment, background fluorescence was measured from the control myocyte (without fura-2 loading), which was similar in size to the myocyte used for the fluorescence recording with identical optical arrangement. The ratio of the fluorescence intensities excited at 340 and 380 nm [*i.e.*, R = F(340)/F(380)] was calculated after subtracting the background fluorescence. The R value was converted to the $[Ca^{2+}]_i$ using the standard equation with parameters for fura-2 fluorescence: R_{min}, R_{max}, β, and K_D.[21] R_{min} and R_{max} values were estimated by a modification of the method of Berlin and Konishi[22] in the cells superfused with the solutions containing ionomycin. The value of β was also estimated in the cells, as described by Bakker *et al.*,[23] from fura-2 fluorescence changes during tetanus. For K_D, the value estimated *in vitro*, 0.24 μM (ionic strength 170 mM, pH 7.2), was used.

Changes in cell length during tetanus were measured with an edge detector (MOPS-SPL-46A001; Hamamatsu Photonics). Myocytes were transilluminated with long-wavelength light (> 600 nm), and cell images were projected onto a linear, 612-element photodiode array. Both edges of the myocyte were detected, and the distance between the edges was measured. Four records of the signals for fluorescence and cell length were averaged.

Prior to the induction of tetanus, myocytes were treated with 0.2 μM thapsigargin (Calbiochem, La Jolla, CA) for 10–15 min to abolish the function of the sarcoplasmic reticulum.[24] This thapsigargin treatment did not affect the resting sarcomere length (SL; ≈1.8 μm).[20] Myocytes were then stimulated by 10-Hz field stimulation for 10 s.[20] The signals for $[Ca^{2+}]_i$ and cell length returned to the baseline values in approximately 40 s in the absence and presence of bupivacaine, and the field stimulation was delivered every minute. As reported previously by us,[20] both signals were stable for more than 1 h, enabling us to examine the effect of bupivacaine without taking into account the effect of the time-dependent rundown in the signals. Changes in fluorescence and cell length were measured simultaneously during tetanus, and an instantaneous plot of $[Ca^{2+}]_i$ *versus* percent cell shortening (Ca-L trajectory) was obtained (a counterclockwise loop). Myofibrillar Ca^{2+} sensitivity was quantitatively estimated by $[Ca^{2+}]_i$ required for 5% shortening from the resting cell length in the falling phase of the Ca-L trajectory (Ca5%).[20] After measuring the Ca-L trajectory under the control condition in the absence of bupivacaine, myocytes were exposed to the solution containing 1 μM and then 3 μM bupivacaine (Sigma), and Ca-L trajectories were obtained.

The experiments using intact cardiomyocytes were performed at 22 ± 2°C.

Preparation of Skinned Ventricular Trabeculae

Skinned ventricular trabeculae were prepared according to our previously reported procedure.[25] The heart was quickly removed from male Wistar rats (weight, 250–350 g; anesthetized with 50 mg/kg intraperitoneal sodium pentobarbital) and perfused with the Ca^{2+}-free Tyrode solution[26] at 30°C. Cylinder-shaped thin trabecular muscles (diameter, 100–150 μm; length, 1–2 mm) were dissected from the right ventricle in the Tyrode solution.

Trabecular muscles were immersed in the relaxing solution [4 mM Mg-adenosine triphosphate (MgATP), 10 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), 10 mM EGTA, 1 mM free Mg^{2+}, 180 mM ionic strength (adjusted by KCl), pH 7.0] containing 1% (vol/vol) Triton X-100 for 60 min at approximately 2°C to disrupt the membrane system. Preparations were then washed in the relaxing solution to remove Triton X-100 and stored at −20°C in the relaxing solution containing 50% (vol/vol) glycerol and 2 mM leupeptin (Peptide Institute, Osaka, Japan) for 1 week or less. Changes in Ca^{2+} sensitivity (pCa_{50}), cooperativity (Hill coefficient), and maximal tension were not noticeable well over 1 week.

Measurement of Isometric Tension

The experimental apparatus has previously been described in detail.[25] Briefly, both ends of the skinned preparation were tied to tungsten wires with a silk thread. One end was connected to a tension transducer (BG-10; Kulite Semiconductor Products, Leonia, NJ) and the other end to a micromanipulator (Narishige, Tokyo, Japan). SL was adjusted to the slack length (1.9 μm) by measuring the laser diffraction in the relaxed condition each time before inducing contraction. Ca^{2+}-activated isometric tension was measured in solutions containing 4 mM MgATP, 10 mM MOPS, 1 mM free Mg^{2+}, various concentrations of free Ca^{2+} [adjusted by Ca/(10 mM EGTA)], 0.1 mM P^1,P^5-di(adenosine5') pentaphosphate, 15 mM creatine phosphate, 15 U/ml creatine phosphokinase at 180 mM ionic strength (adjusted by KCl) (pH 7.0).

Maximal Ca^{2+}-activated tension was measured according to a previously described procedure.[27] Just prior to contraction (pCa 4.8), the preparation was bathed in the low-EGTA (1 mM) relaxing solution for approximately 15 s to minimize the buffering effect of EGTA (the low-EGTA relaxing solution was used only for this purpose). Contraction was stopped by transferring the preparation to the relaxing solution containing 10 mM EGTA. This procedure was repeated in the presence of 1, 10, and 100 μM bupivacaine (in this order).

For the estimation of myofibrillar Ca^{2+} sensitivity, we measured the pCa–tension relations in the absence and presence of bupivacaine (10 and 100 μM). pCa–tension relations were obtained by cumulatively increasing the free Ca^{2+} concentration from the relaxed condition (pCa $>$ 9) to the maximally activated condition (pCa 4.8).[25] First, the pCa–tension relation was obtained during the control condition in the absence of bupivacaine. This tension measurement was repeated in the presence of 10 and then 100 μM bupivacaine. Each pCa–tension relation was fitted to the Hill equation:

$$\log[P/(100 - P)] = n_H[pCa_{50} - pCa]$$

where P is the relative tension expressed as a percentage of the maximum (pCa 4.8), n_H is the Hill coefficient, and pCa_{50} is $-\log [Ca^{2+}]$ at P = 50%.[25]

At the end of experiment, the preparation was activated in the absence of bupivacaine for measurement of maximal tension or Ca^{2+} sensitivity during the control condition.

We further examined the effect of bupivacaine on tension induced by rigor cross-bridges in the absence of Ca^{2+} (Ca^{2+}-independent tension). In this experiment, the skinned preparation was first bathed in the relaxing solution, and the MgATP concentration was gradually decreased to induce tension.[28,29] The control pMgATP–tension relation was first obtained in the absence of bupivacaine. Then, the pMgATP–tension relation was measured in the presence of 10 and 100 μM bupivacaine. As was done for the measurement of Ca^{2+}-activated tension, SL was adjusted to 1.9 μm in the relaxing solution before inducing tension. The pMgATP–tension relation was fitted to the Hill equation using the values of n_H and the midpoint of the relation ($pMgATP_{50}$).

For the estimation of tension per cross-sectional area, the diameter of muscle was measured in the relaxed condition using a microscope (SMZ645; Nikon) at a magnification of 225×.[25]

All experiments using skinned ventricular trabeculae were performed at 20.0 ± 0.2°C.[25–27]

Statistical Analysis

The effects of bupivacaine were assessed using one-way analysis of variance with repeated measures and the Scheffé *post hoc* test. Comparisons between control values obtained at the beginning and the end of the studies with skinned preparations were made using Student *t* test for paired data. Statistical significance was verified at $P < 0.05$ in all cases. Correlation between pCa_{50} and maximal tension was evaluated by testing the correlation of these parameters.[27] All data are expressed as mean ± SEM, with n representing the number of muscles.

Results

Effect of Bupivacaine on Myofibrillar Ca^{2+} Sensitivity in Isolated Intact Cardiomyocytes

First, we confirmed that bupivacaine at the concentrations of 1 and 3 μM did not affect the fluorescence signals of fura-2 in the presence of various concentrations of free Ca^{2+} (pCa from $>$ 9 to 4.3) without myocytes. The baseline values for $[Ca^{2+}]_i$ (*i.e.*, during diastole) were not significantly different between groups ($P > 0.05$; n = 5), *i.e.*, 58.68 ± 5.31, 57.56 ± 14.21, and 84.64 ± 19.41 nM, respectively, in the absence and presence of 1 and 3 μM bupivacaine. Likewise, the diastolic cell length was not significantly affected by bupivacaine in that the values were 135.57 ± 4.51, 135.48 ± 4.89, and 135.27 ± 5.03 μm in the absence and presence of 1 and 3 μM bupivacaine ($P > 0.05$; n = 5), respectively.

Figures 1A and B show typical recordings for the time course of changes in $[Ca^{2+}]_i$ and cell shortening, respectively, during tetanus obtained during the control condition in the absence of bupivacaine, compared with those in the presence of 1 and 3 μM bupivacaine. It was found that bupivacaine suppressed an increase in $[Ca^{2+}]_i$ and subsequent cell shortening, with the magnitude being greater for cell shortening (figs. 1A and B). Figure 1C shows an instantaneous plot of $[Ca^{2+}]_i$ *versus* cell shortening (*i.e.*, Ca-L trajectory) taken from the data of figures 1A and B. Consistent with our previous report,[20] during the control condition, the Ca-L trajectory followed nearly the same path during shortening and relengthening. It is clearly seen that the Ca-L trajectory is shifted to the right (*i.e.*, higher $[Ca^{2+}]_i$ side) and maximal cell shortening

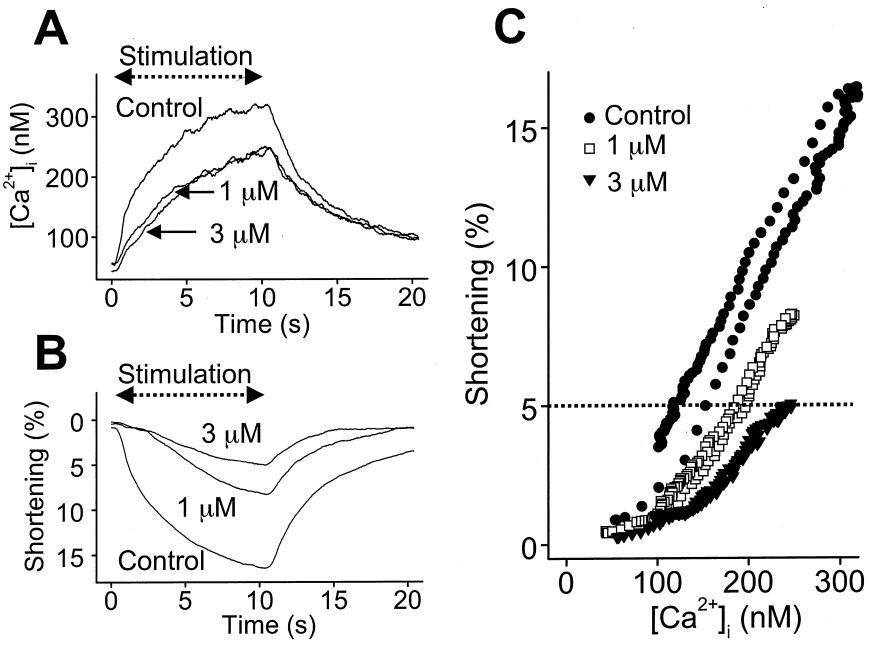

Fig. 1. Typical chart recordings for changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (*A*) and percent cell shortening (*B*) during tetanus in isolated rat ventricular myocytes in the absence and presence of 1 and 3 μM bupivacaine. Control = in the absence of bupivacaine. Myocytes were tetanized for 10 s after treatment with thapsigargin (indicated by "Stimulation"). The same myocyte was used to test the effect of bupivacaine. Data of four signals were averaged. (*C*) An instantaneous plot of $[Ca^{2+}]_i$ *versus* cell shortening (*i.e.*, Ca-L trajectory). Data were taken from (*A*) and (*B*). Dotted line shows 5% cell shortening.

(reached during the 10-s tetanus) is depressed with the addition of bupivacaine. The former is opposite to the effect of EMD 57033, a Ca^{2+} sensitizer, and shows a decrease in Ca^{2+} sensitivity of myofilaments.[20] The effect of bupivacaine on myofibrillar Ca^{2+} sensitivity was quantitatively assessed by $[Ca^{2+}]_i$ required for 5% cell shortening (*i.e.*, Ca5%; dotted line in fig. 1C).[20] Bupivacaine increased Ca5% with a statistically significant effect at 3 μM (fig. 2).

Effect of Bupivacaine on Ca^{2+}-activated Isometric Tension in Skinned Ventricular Muscle

Figure 3 shows the effect of bupivacaine on maximal Ca^{2+}-activated tension obtained at pCa 4.8 in skinned ventricular trabeculae. During the control condition in the absence of bupivacaine, maximal tension was 46.34 ± 2.96 kN/m^2, similar to what was obtained in our previous studies.[25–27] Bupivacaine was found to significantly decrease maximal tension at 10 and 100 μM.

Figure 4 shows typical chart recordings for changes in isometric tension obtained by varying the free Ca^{2+} concentration (pCa from > 9 to 4.8) in the absence and presence of bupivacaine (100 μM). In the absence of bupivacaine, the pCa–tension relation (pCa_{50} and n_H)

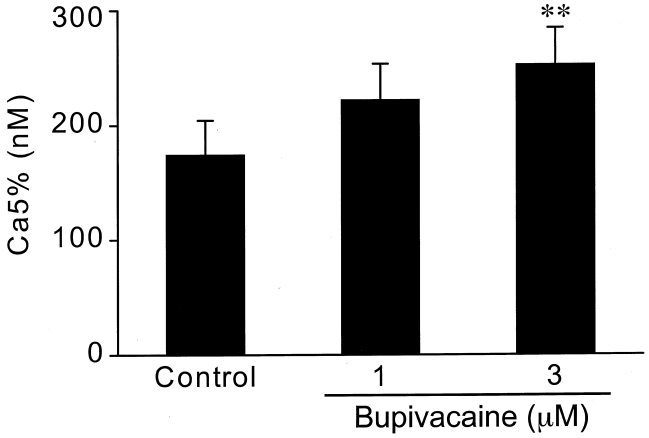

Fig. 2. Effect of bupivacaine on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) required for 5% cell shortening (Ca5%). Control = in the absence of bupivacaine. The values were significantly different as follows: 3 μM *versus* control ($P < 0.01$), 3 μM *versus* 1 μM ($P < 0.05$). Asterisks indicate the significant difference compared with control for 3 μM ($P < 0.01$). Vertical bars are SEM of five data points.

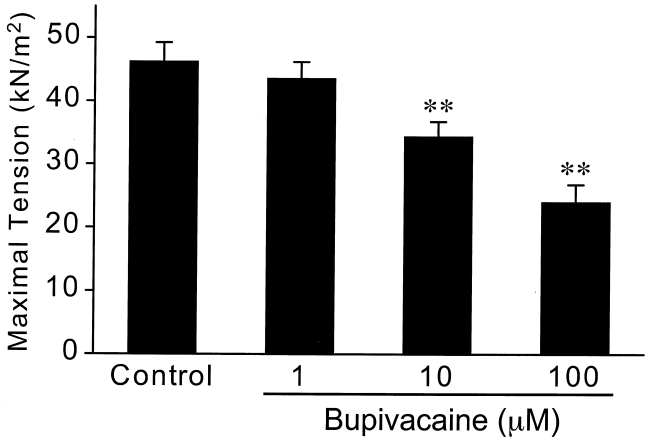

Fig. 3. Effect of bupivacaine on maximal Ca^{2+}-activated tension in skinned ventricular trabeculae obtained at pCa 4.8. The concentration of bupivacaine was changed from 1 to 100 μM. Control = in the absence of bupivacaine. The values were significantly different as follows ($P < 0.01$ for all): 10 μM *versus* control, 10 μM *versus* 1 μM, 100 μM *versus* control, 100 μM *versus* 1 μM, and 100 μM *versus* 10 μM. Asterisks indicate the significant difference compared with control ($P < 0.01$). Tension at pCa 4.8 was regarded as the maximum because a further increase in the Ca^{2+} concentration had no effect on isometric tension in the presence of 100 μM bupivacaine. Vertical bars are SEM of eight data points.

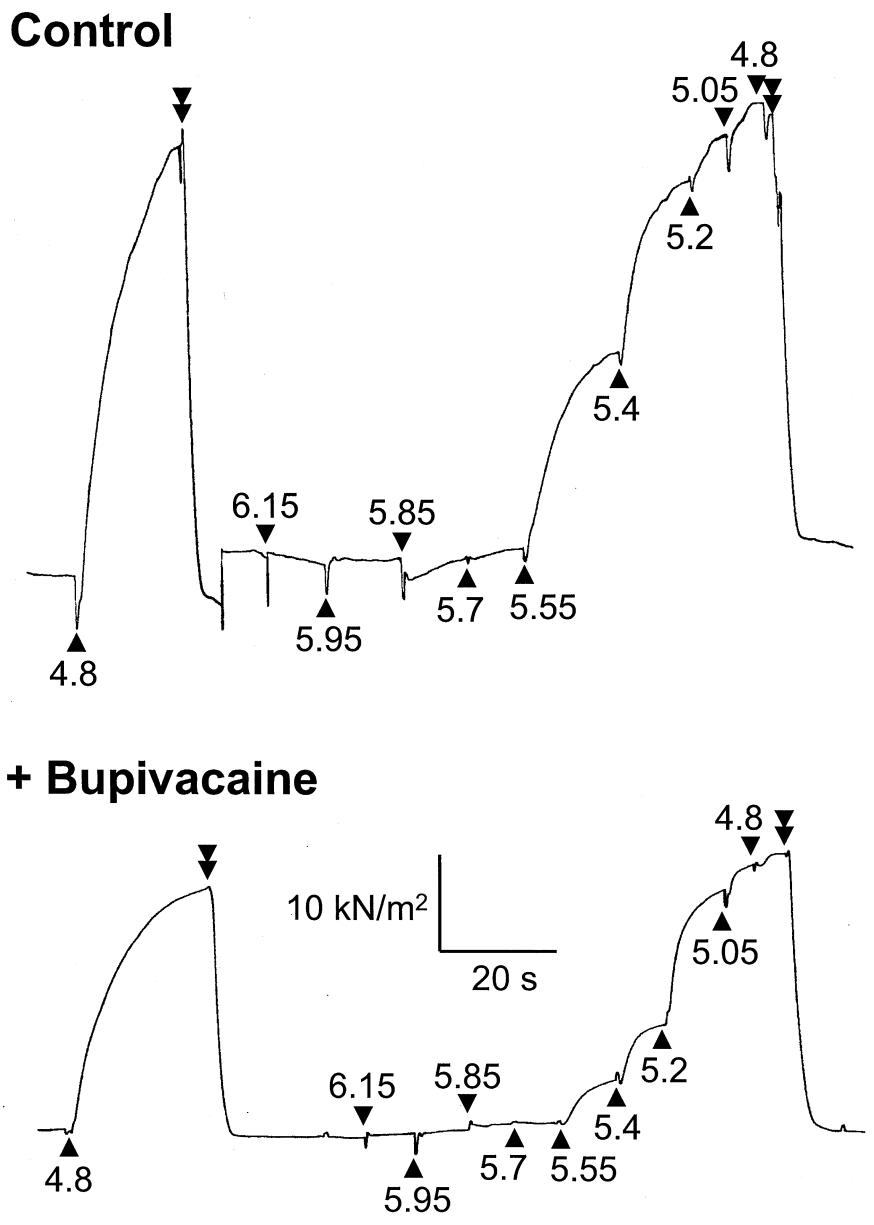

Fig. 4. Typical chart recordings for measurement of isometric tension with increasing the concentration of free Ca^{2+} in the absence (control) and presence of 100 μM bupivacaine in skinned ventricular trabeculae. pCa was varied from greater than 9 (relaxed condition) to 4.8 in a stepwise manner. Note that the final tension at pCa 4.8 is similar to that obtained before varying pCa in the absence and presence of bupivacaine, showing reproducibility of tension development. Arrowheads indicate the points at which muscle was transferred to the solution containing a higher concentration of free Ca^{2+}, as indicated. Double arrowheads indicate the points at which muscle was transferred to the relaxing solution. Data taken from the same preparation.

and maximal tension were not significantly altered on repeated activations (experiment repeated four times; N. Fukuda, Ph.D., unpublished data, September 16, 1999). In the presence of bupivacaine, maximal tension (pCa 4.8) was lower and its inhibitory effect was more pronounced during submaximal activation (pCa 5.55 and 5.4). Figure 5A shows the pCa–tension relations (of which the ordinate is expressed in absolute values of active tension, kN/m^2) in the absence and presence of 10 and 100 μM bupivacaine. It is clearly seen that bupivacaine significantly suppresses Ca^{2+}-activated tension over the range of pCa used (*i.e.*, from 5.7 to 4.8). Absolute values of tension at various pCa values were converted to relative values (*i.e.*, relative to the maximum at pCa 4.8) to obtain the normalized pCa–tension relations (fig. 5B). We found that the pCa–tension relation was significantly shifted to the right (*i.e.*, the lower pCa [higher free Ca^{2+} concentration] side) with 10 and 100 μM bupivacaine, showing a decrease in myofibrillar Ca^{2+} sensitivity (table 1). The Hill coefficient (n_H) values for the pCa–tension relations are summarized in table 1. Bupivacaine showed a tendency to decrease n_H, but not significantly.

It was found that a highly correlated linear relation ($R = 0.81$, $P < 0.0005$) was present between pCa_{50} and maximal Ca^{2+}-activated tension in preparations used for the measurement of the pCa–tension relations (fig. 5C).

After using bupivacaine (up to 100 μM), we measured maximal tension (fig. 3) or the pCa–tension relation (fig. 5) in the absence of bupivacaine. Skinned preparations were immersed in the relaxing solution to wash out bupivacaine for 20 min. The observed value for maximal tension was 44.89 ± 3.02 kN/m^2 which was comparable to that obtained in the beginning of the experiment

($P > 0.05$ compared with the control value in fig. 3; *i.e.*, 46.34 ± 2.96 kN/m^2). The value of pCa_{50} was 5.47 ± 0.02 and that of n_H was 5.10 ± 0.49 ($P > 0.05$ for each parameter compared with a corresponding control value in table 1). These results suggest that the inhibitory effect of bupivacaine on contractile proteins is reversible, in contrast to its blocking effect on sodium channels.[8]

Table 1. Summary of pCa_{50} and n_H Values for pCa-Tension Relation in Figure 5

	pCa_{50}	n_H
Control	5.49 ± 0.02	5.05 ± 0.43
Bupivacaine		
10 μM	5.42 ± 0.01	4.82 ± 0.61
100 μM	5.36 ± 0.02	4.60 ± 0.48

The pCa_{50} values were significantly different between groups as follows (n = 5): 10 μM *versus* control ($P < 0.05$), 100 μM *versus* control ($P < 0.01$), 100 μM *versus* 10 μM ($P < 0.05$). The n_H values were not significantly different between groups ($P > 0.05$).

pCa_{50} = −log [Ca^{2+}] at P = 50%; n_H = Hill coefficient.

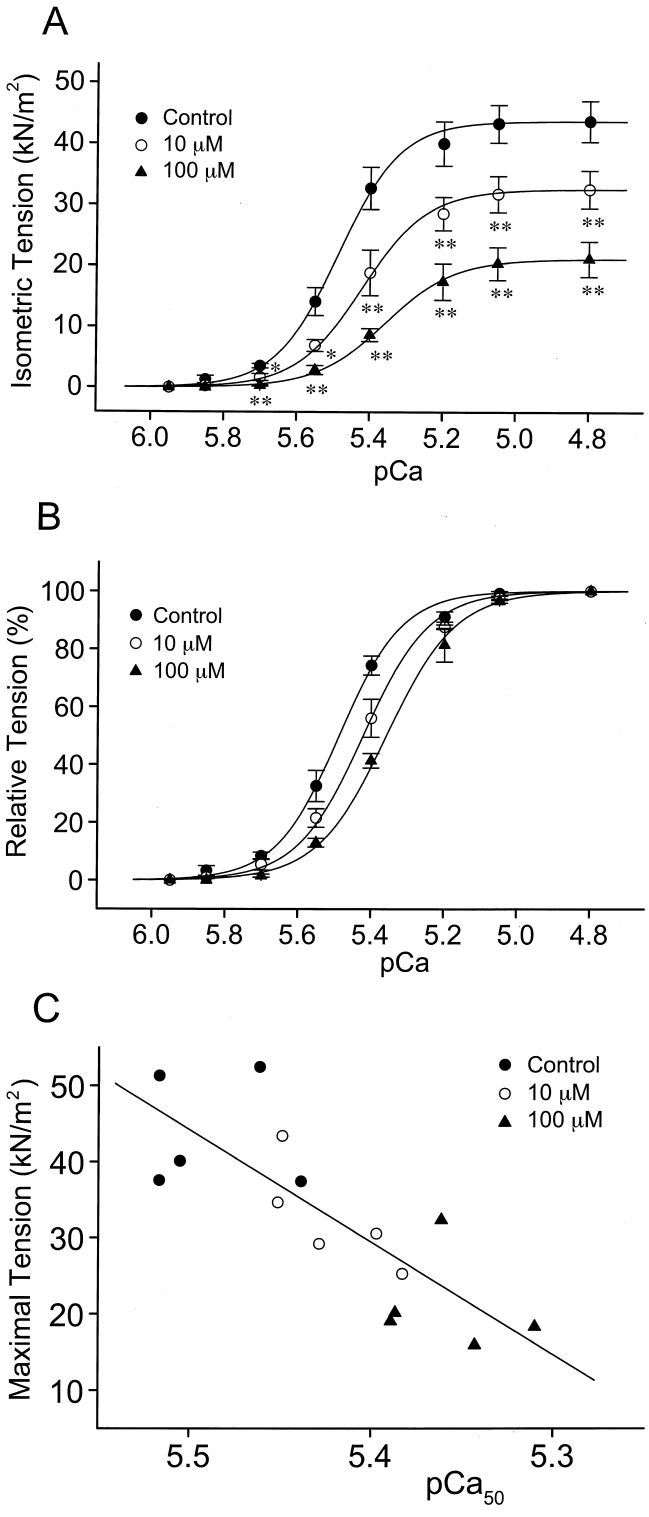

Fig. 5. Effect of bupivacaine (10 and 100 μM) on Ca^{2+}-activated tension in skinned ventricular trabeculae. (*A*) pCa–tension relations in the absence and presence of bupivacaine. Results are shown in absolute values of tension. Control = in the absence of bupivacaine. Asterisks indicate significant differences compared with control for 10 and 100 μM bupivacaine (*$P < 0.05$; **$P < 0.01$). (*B*) Same as in (*A*) but tension was normalized with respect to that at pCa 4.8 for all curves. Data obtained for each preparation were fitted to the Hill equation with the mean values of pCa_{50} and n_H [isometric tension obtained at pCa 4.8 was regarded as the maximum for each curve in (*A*)]. Vertical bars are SEM of five data points. (*C*) The linear regression line between the midpoint of the pCa–tension relation (pCa_{50}) and maximal Ca^{2+}-activated tension at pCa 4.8 (data were taken from the same preparations used for measurement of the pCa–tension relations). A high correlation (R = 0.81; $P < 0.0005$) was obtained.

Effect of Bupivacaine on Tension Induced by Rigor Cross-bridges in the Absence of Ca^{2+}

Figure 6 shows typical changes in tension induced by lowering the MgATP concentration in the absence and presence of 100 μM bupivacaine. During the control condition in the absence of bupivacaine, tension started to develop by lowering the MgATP concentration to 5.25, reached a plateau at pMgATP 5.55, then gradually declined with a further decrease in the MgATP concentration.[28,29] In the same preparation, the shape of the relation between pMgATP and tension (*i.e.*, the pMgATP–tension relation) did not change in the second series of tension measurement. We found that in the presence of bupivacaine, maximal tension was lower and its inhibitory effect was substantially more pronounced during submaximal activation (see pMgATP 5.25 in fig. 6). Figure 7 shows the effect of 10 and 100 μM bupivacaine on the pMgATP–tension relation normalized to maximal tension. The midpoint of the pMgATP–tension relation ($pMgATP_{50}$) was similar to what was previously reported using rat ventricular muscle (table 2).[28] It was found that bupivacaine significantly shifted the curve to the left, *i.e.*, to the lower MgATP concentration side, and slightly decreased the n_H value ($P > 0.05$; table 2). As found for Ca^{2+}-activated tension (figs. 3 and 5A), bupivacaine significantly decreased Ca^{2+}-independent tension at maximal activation (fig. 7).

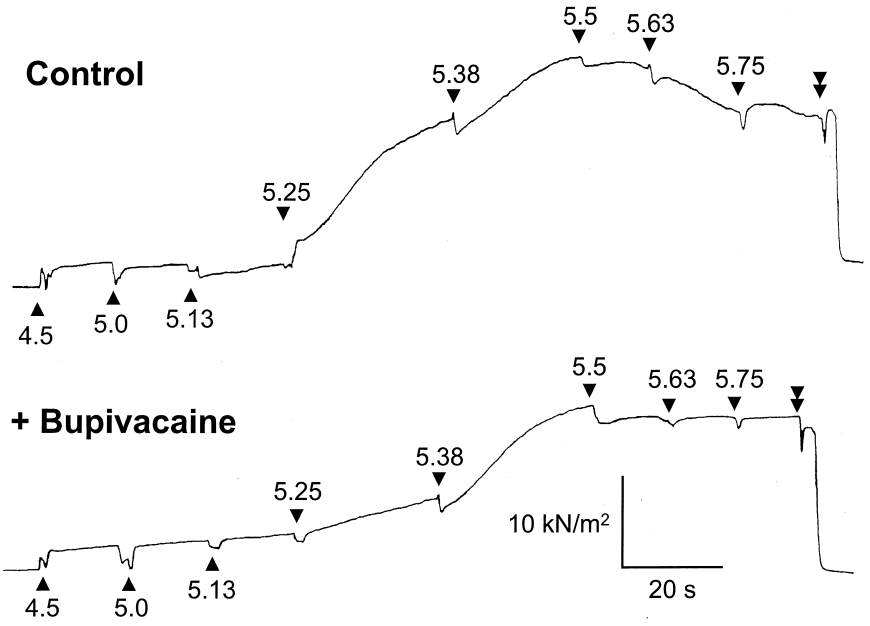

Fig. 6. Typical chart recordings for the measurement of isometric tension induced by rigor cross-bridges in the absence (control) and presence of 100 μM bupivacaine in skinned ventricular trabeculae. Ca^{2+} was absent. Tension was induced by gradually reducing the Mg-adenosine triphosphate (MgATP) concentration. Arrowheads indicate the points at which muscle was transferred to the solution containing a lower concentration of MgATP, as indicated. Double arrowheads indicate the points at which muscle was transferred to the relaxing solution. The same preparation was used.

We confirmed that $pMgATP_{50}$ and maximal tension were not significantly affected by increasing the creatine phosphokinase concentration from 15 to 150 U/ml. This indicates that 15 U/ml creatine phosphokinase was sufficient to remove adenosine diphosphate in the myofilament lattice, and thus the effect of adenosine diphosphate on cross-bridges[30,31] could be disregarded in our experimental conditions.

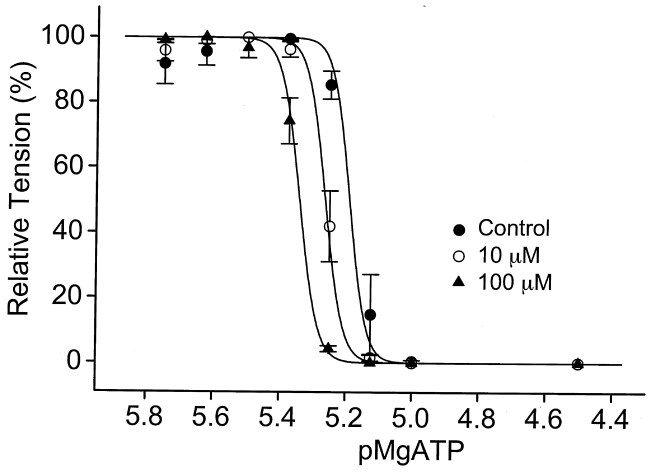

Fig. 7. Effect of bupivacaine (10 and 100 μM) on Ca^{2+}-independent active tension. Results are shown in pMg-adenosine triphosphate (pMgATP)–tension relations. Tension was normalized with respect to maximal tension at pMgATP 5.5, 5.5, and 5.63, respectively, in the absence (control) and presence of 10 and 100 μM bupivacaine. Data obtained for each preparation was fitted to the Hill equation with the mean values of $pMgATP_{50}$ and n_H. Absolute values of maximal tension were 25.24 ± 3.61, 20.56 ± 3.37, and 15.09 ± 2.89 kN/m^2, respectively, in the absence (control) and presence of 10 and 100 μM bupivacaine, and the values were significantly different as follows: 10 μM *versus* control ($P < 0.05$), 100 μM *versus* control ($P < 0.01$), and 100 μM *versus* 10 μM ($P < 0.05$). Data obtained for each preparation were fitted to the Hill equation with the mean values of $pMgATP_{50}$ and n_H. Vertical bars are SEM of five data points.

Discussion

The findings of the current study are threefold. First, bupivacaine decreased Ca^{2+} sensitivity of myofilaments in isolated intact ventricular myocytes. Second, bupivacaine decreased myofibrillar Ca^{2+} sensitivity, as well as maximal Ca^{2+}-activated tension, in skinned ventricular trabeculae. Third, bupivacaine suppressed tension induced by lowering the MgATP concentration in the absence of Ca^{2+} in skinned ventricular trabeculae.

In the current study, we induced tetanic contractions in thapsigargin-treated isolated cardiomyocytes and confirmed the result of our previous study,[20] that the loop of the Ca-L trajectory is very narrow (fig. 1C) as compared with the phase-plane loop of twitch contraction.[32] This suggests that the dynamic equilibrium between $[Ca^{2+}]_i$ and cell length is achieved during shortening and relengthening, presumably owing to slow changes in $[Ca^{2+}]_i$ during tetanus.[20] It is therefore considered that the Ca-L trajectory used in this study is suitable to examine the effects of various compounds on steady state Ca^{2+} sensitivity of myofilaments in intact myocytes. Our data showed that bupivacaine suppressed the increase of

Table 2. Summary of $pMgATP_{50}$ and n_H Values for pMgATP-Tension Relation in Figure 7

	$pMgATP_{50}$	n_H
Control	5.19 ± 0.02	17.93 ± 1.49
Bupivacaine		
10 μM	5.26 ± 0.02	16.80 ± 2.04
100 μM	5.34 ± 0.01	16.20 ± 1.98

The pMgATP values were significantly different between groups as follows (n = 5): 10 μM *versus* control ($P < 0.05$), 100 μM *versus* control ($P < 0.01$), 100 μM *versus* 10 μM ($P < 0.05$). The n_H values were not significantly changed ($P > 0.05$).

pMgATP = −log magnesium-adenosine triphosphate; n_H = Hill coefficient.

$[Ca^{2+}]_i$ and subsequent cell shortening (figs. 1A and B), and its effect was greater on shortening, as judged by the rightward shift of the Ca-L trajectory (fig. 1C; see also Fig. 2 for quantitative analysis). Previous reports suggest that the acute inhibitory effect of bupivacaine on systolic $[Ca^{2+}]_i$ is primarily attributable to its blocking effect on sarcolemmal Na^+ and Ca^{2+} channels and also to its inhibitory effect on the sarcoplasmic reticulum function.[8–10,12,13] In our experimental condition where tetanus was induced, however, the observed decrease in systolic $[Ca^{2+}]_i$ (fig. 1A) likely arose in large part from the blocking effect of bupivacaine on sarcolemmal Ca^{2+} channels. On the other hand, the rightward shift of the Ca-L trajectory (especially a significant increase in the Ca5% value at 3 μM; fig. 2) shows that bupivacaine decreased myofibrillar Ca^{2+} sensitivity in intact cardiac muscle. We therefore consider that bupivacaine exerts a negative inotropic effect *via*, at least in part, inhibition of the pathway beyond intracellular Ca^{2+} mobilization by the membrane system.

Our results with skinned cardiac muscle showed that bupivacaine shifted the pCa–tension relation to the right and concomitantly decreased maximal Ca^{2+}-activated tension at the concentrations of 10 μM and higher (figs. 3 and 5A and B; table 1). Herzig *et al.*[3] reported that 10 μM bupivacaine decreased twitch tension by approximately 20% in guinea pig cardiac muscle. The experimental condition of this earlier study is different from ours, where atrial, but not ventricular, muscle of different animal species was used; therefore, care should be taken in interpreting the experimental data. Nevertheless, it is safe to consider that, looking at the data for 10 μM bupivacaine in figures 3, 5A, and 7, the result of the current study is in reasonable agreement with that of Herzig *et al.*[3] In a different series of experiments, we confirmed that 10 μM bupivacaine depressed peak twitch tension by approximately 25% and abbreviated time required for relaxation from peak tension to 50% tension by approximately 10% in isometrically contracting ventricular papillary muscle of the rat (unpublished data, July 15, 2000, Yasushi Mio, M.D., Tokyo, Japan).

One straightforward interpretation for the decrease in Ca^{2+} sensitivity is that bupivacaine somehow inhibits the binding of Ca^{2+} to troponin C (TnC) by directly acting on TnC (or other subunits of the troponin molecule, *i.e.*, troponin I and troponin T) and, consequently, decreases isometric tension during submaximal activation. However, it should be emphasized that bupivacaine suppressed not only Ca^{2+} sensitivity, but also maximal Ca^{2+}-activated tension (figs. 3, 4, and 5A), and its inhibitory effect on Ca^{2+} sensitivity and that on maximal tension were closely correlated (fig. 5C). This is consistent with the result of the experiment with intact myocytes in that bupivacaine also depressed maximal cell shortening (reached during the 10-s tetanus; fig. 1C). These results favor the interpretation that a direct interaction of bupivacaine at a locus beyond Ca^{2+} binding to TnC, possibly the interaction of myosin molecules with actin, underlies the observed negative inotropic effect of this compound at the myofilament level.

It is well established that decreasing the MgATP concentration in the absence of Ca^{2+} promotes the formation of rigor cross-bridges, which suppresses the inhibition of the regulatory proteins troponin and tropomyosin on thin filaments just as if Ca^{2+} were bound to TnC.[33] It has been reported that these rigor cross-bridges, formed on decrease in the MgATP concentration, enhance the formation of the "on" state of thin filaments, which subsequently accelerates the attachment of neighboring myosins to thin filaments and therefore induces tension.[29] A further decrease in the MgATP concentration resulted in a decrease in tension (figs. 6 and 7), presumably because the number of actively cycling (force-generating) cross-bridges is decreased. Here, tension is a function of the number of force-generating and rigor cross-bridges as well as the relative activation of thin filaments.[34] In the current study, bupivacaine substantially shifted the pMgATP–tension relation to the higher pMgATP (*i.e.*, lower MgATP concentration) side and also decreased maximally activated tension (figs. 6 and 7; table 2). These results appear to provide further evidence that bupivacaine can suppress the pathway downstream from Ca^{2+} binding to TnC, possibly the actomyosin interaction *per se*. Therefore, based on the result of Ca^{2+}-independent activation (figs. 6 and 7), as well as that of maximal Ca^{2+}-activated tension (figs. 3, 4, and 5A), we propose that bupivacaine can directly act on the actomyosin interaction, thereby either decreasing the number of force-generating cross-bridges or reducing the force per force-generating cross-bridge, or both, which results in a decrease in apparent Ca^{2+} sensitivity of myofilaments.

Boban *et al.*[2] reported that bupivacaine at the concentrations of 3–5 μM reduced ventricular systolic pressure by 20–30% in isolated guinea pig hearts. Considering a similar concentration range used therein, our proposed mechanism, albeit the possible species difference (rats *vs.* guinea pigs), may in part underlie the cardiodepressant effect of bupivacaine demonstrated in isolated whole hearts.[2]

It is widely accepted that n_H of the pCa–tension relation and that of the pMgATP–tension relation reflect the cooperative activation of thin filaments.[28,29,31,35] Bupivacaine showed a tendency to slightly reduce cooperativity both in Ca^{2+}-dependent (table 1) and Ca^{2+}-independent activation (table 2). Here, it is unlikely that bupivacaine directly affects cooperative activation of thin filaments by specifically acting on thin filament proteins (*i.e.*, troponin, tropomyosin, or actin) as the magnitude of the reduction of cooperativity was very small (approximately 10%) and the effect was statistically not significant ($P > 0.05$) both in Ca^{2+}-dependent and

-independent activation (see 100 μM in tables 1 and 2). It is well established that strongly binding cross-bridges such as force-generating and rigor cross-bridges enhance the cooperative activation of thin filaments.[25,31,36] We therefore consider that the tendency of reduced cooperativity observed in the presence of bupivacaine is coupled with a decrease in the number of strongly binding cross-bridges or a reduction of the force per strongly binding cross-bridge, or both, rather than its direct effect on thin filament proteins.

We noted a difference regarding the Ca^{2+}-desensitizing effect of bupivacaine in isolated intact cardiomyocytes and in skinned trabeculae. In intact cells, 1 and 3 μM bupivacaine altered the shape and position of the Ca-L trajectory (with statistically significant effect at 3 μM; figs. 1C and 2), whereas 10 μM or higher was necessary to lower isometric tension in skinned preparations (figs. 3 and 5A; table 1). The following two hypotheses could be raised regarding the greater effect of bupivacaine in intact cells. First, during shortening of tetanized cells, SL continues to decrease from the resting value (*i.e.*, approximately 1.8 μm) until stimulation is terminated. On the other hand, changes in SL (adjusted to 1.9 μm in the relaxed condition) are reportedly minimal during isometric contraction in rat skinned ventricular trabeculae.[27,37] It is known that a decrease in SL from approximately 1.8 μm results in an increased double-overlap region of the thick and thin filaments[38] as well as widening of the lateral separation of these filaments,[39] both of which probably reduce the likelihood of myosin attaching to the thin filament. Therefore, provided that bupivacaine directly acts on the actomyosin interaction as discussed above, its effect is more pronounced during cell shortening, where cross-bridge formation is relatively retarded because of progressive SL shortening, compared with during isometric contraction (see Fukuda *et al.*[26] for a similar SL-dependent effect of hydrogen ion or inorganic phosphate). Second, in intact preparations, bupivacaine may influence the intracellular signal transduction pathway, such as by deactivating myosin light chain kinase[40] or protein kinase C[41] (resulting in dephosphorylation of myosin light chain 2, *i.e.*, decreased Ca^{2+} sensitivity[42]), or change the intracellular milieu, such as by lowering the intracellular pH, and subsequently enhance its negative inotropic effect. Here it should be emphasized that the protein kinase A–dependent phosphorylation of troponin I (resulting in a decrease in Ca^{2+} sensitivity[43]) is not likely involved in the Ca^{2+}-desensitizing effect of bupivacaine as this compound is known to inhibit the production of cyclic-3',5'-adenosine monophosphate.[44]

The literature indicates that, in humans, the plasma concentration of bupivacaine is in the range of 1–3 μg/ml (approximately 3–10 μM) with typical clinical doses.[45,46] It is well known that an accidental rapid intravenous injection of bupivacaine can lead to cardiac arrest. In a recent study, Chang *et al.*[6] reported that an intracoronary administration of bupivacaine induced ventricular fibrillation in conscious sheep (4 of 6 animals). The authors also noted that, in the surviving animals, bupivacaine produced a decrease in myocardial contractility (as indexed by dP/dt_{max}), tachycardia (probably due to baroreceptor reflex activation), and widening of electrocardiographic QRS complexes. It is likely that the well-documented arrhythmogenic effects of bupivacaine[4–6] are due to reentrant phenomena caused by drug-induced conduction disorders.[5] On the other hand, our results clearly show that bupivacaine at micromolar concentrations to 10 μM decreases Ca^{2+} sensitivity in isotonically (figs. 1 and 2) as well as in isometrically (figs. 3, 5, and 7; tables 1 and 2) contracting ventricular preparations. Reportedly, in rats, cardiac arrest is induced by an intravenous infusion of bupivacaine at the plasma concentration of approximately 300 μM.[47] The possible species difference (rats *vs.* humans) should be taken into account in interpreting the current data; however, our results suggest that clinically relevant doses of bupivacaine may suppress the contractility of cardiac muscle at the myofilament level *in vivo*. It is therefore reasonable to conclude that clinically relevant doses of bupivacaine may reduce cardiac contractility and that an accidental rapid intravenous injection of this compound may lead to cardiac arrest at the myofilament level in humans.

In several aspects, however, the conditions of our experiments were obviously different from those encountered in humans, and care should be taken in the interpretation the experimental data. One of the important viabilities may be the temperature, since some volatile anesthetics (*i.e.*, halothane, isoflurane, and enflurane)[48] and lidocaine–lignocaine[49] reportedly show temperature-dependent differences in their negative inotropic effects. We performed all experiments at 20–22°C to match the experimental conditions to those established in our previous studies.[20,25–27] The negative inotropic effect of bupivacaine may depend on temperature, and thus future studies are needed to clarify the temperature dependence of its action in an experimental system (such as in isometrically contracting ventricular papillary muscle) that allows us to use a physiologically more relevant temperature.

In the current study, we demonstrated that bupivacaine decreased Ca^{2+} sensitivity of myofilaments in intact and skinned ventricular muscle of the rat. In skinned muscle preparations, bupivacaine suppressed Ca^{2+}-independent tension as well as maximal Ca^{2+}-activated tension, suggesting that it directly inhibits the actomyosin interaction, and myofibrillar Ca^{2+} sensitivity is thereby apparently decreased. This effect may in part give rise to the overall cardiodepressant action of bupivacaine *in vivo*.

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