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Bupivacaine Inhibition of L-Type Calcium Current in Ventricular Cardiomyocytes of Hamster

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***Background*: The local anesthetic bupivacaine is cardiotoxic when accidentally injected into the circulation. Such cardiotoxicity might involve an inhibition of cardiac L-type Ca^{2+} current ($I_{Ca,L}$). This study was designed to define the mechanism of bupivacaine inhibition of $I_{Ca,L}$.**

***Methods*: Cardiomyocytes were enzymatically dispersed from hamster ventricles. Certain voltage- and time-dependencies of $I_{Ca,L}$ were recorded using the whole-cell patch clamp method in the presence and absence of different concentrations of bupivacaine.**

***Results*: Bupivacaine, in a concentration-dependent manner (10–300 μM), tonically inhibited the peak amplitude of $I_{Ca,L}$. The inhibition was characterized by an increase in the time of recovery from inactivation and a negative-voltage shift of the steady-state inactivation curve. The inhibition was shown to be voltage-dependent, and the peak amplitude of $I_{Ca,L}$ could not be restored to control levels by a wash from bupivacaine.**

***Conclusions*: The inhibition of $I_{Ca,L}$ appears, in part, to result from bupivacaine predisposing L-type Ca channels to the inactivated state. Data from washout suggest that there may be two mechanisms of inhibition at work. Bupivacaine may bind with low affinity to the Ca channel and also affect an unidentified metabolic component that modulates Ca channel function. (Key words: Anesthetic, local: bupivacaine. Hamster, heart; cardiomyocytes. Voltage clamp; whole-cell patch clamp; L-type calcium current.)**

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WHEN bupivacaine is accidentally injected into the circulation, cardiovascular complications such as dysrhythmias and cardiac arrest may ensue.[1] Total blood concentrations of bupivacaine in patients with these complications typically range from 2-4 μg/ml.[2] Cardiodepressant effects of bupivacaine are seen *in vitro* by reductions in contraction force of heart tissue.[3-7] A determinant of the cardiodepression might be an inhibition of calcium current through L-type Ca^{2+} channels ($I_{Ca,L}$). Electrophysiologic studies have examined the effect of bupivacaine on $I_{Ca,L}$ in rat sensory neurons[8] and in cardiac cells using calcium-dependent action potentials in myocardium of guinea pig,[6,9,10] double sucrose gap voltage clamp of frog atrial tissue,[11] and whole-cell patch clamp of isolated ventricular cardiomyocytes of guinea pig.[9,12] All report that bupivacaine inhibits the current, but few details are available that disclose the mechanism of inhibition. With this in mind, we have studied certain characteristics of the bupivacaine inhibition of $I_{Ca,L}$, such as concentration-response, steady-state inactivation, inactivation time constants, time course of recovery from inactivation, and voltage dependency.

Methods and Materials

Preparation of Cardiomyocytes

Cardiomyocytes were isolated from the left ventricular free wall of hearts of 16 normal male hamsters (aged 92 to 199 days) by a protocol approved by the Institutional Animal Care and Use Committee of Nassau County Medical Center. Hamsters, BIO F1B strain (BIO-Breeders, Inc.,Watertown, MA), were anesthetized by intraperitoneal injection of pentobarbital sodium (60 mg/kg). When corneal and footpad-withdrawal reflexes were absent the anesthetized hamster was killed by cervical dislocation. The heart was quickly removed intact with a small length of aorta. Cardiomyocytes were isolated by an enzymatic dispersion method slightly modified from that described elsewhere.[13] Briefly, the

aorta was cannulated on a Langendorff column and perfused for 15 min with nominally Ca^{2+}-free (10–20 μM Ca^{2+}) modified Tyrode solution that contained (mM) 135 NaCl, 5.4 KCl, 1 $MgCl_2$, 10 HEPES (N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]), 0.33 NaH_2PO_4, 10 glucose (pH 7.38–7.39 with NaOH). Perfusion with an enzyme solution, containing 0.09% collagenase (Boehringer Mannheim, type B) and from 0.0047 to 0.019% protease (Sigma, type XIV) mixed in nominally Ca^{2+}-free Tyrode solution, followed for 15–20 min. The softened heart was perfused for 4–5 min with a modified "KB medium"[14] that contained (mM) 30 KCl, 30 KH_2PO_4, 3 $MgSO_4$, 50 glutamate, 20 taurine, 10 glucose, 0.5 EGTA (Ethylene Glycol-bis[b-Aminoethyl Ether]N,N,N',N'-Tetraacetic Acid), 20 HEPES (pH 7.3 with KOH). All perfusates were heated to 37°C and oxygenated (100% O_2). The heart was removed from the column, and the left ventricular free wall was dissected in modified KB solution. Gentle agitation with forceps dispersed the cells. Filtration through a 200-μm nylon mesh (Tetko Inc., Elmsford, NY) separated individual cardiomyocytes from tissue clumps. Myocytes were incubated for a minimum of 1 h in modified KB solution at room temperature and stored at 10°C for use during the same day. Approximately 80–90% of the myocytes survived in modified KB solution. Survival was appraised by the retention of rod shape and distinct sarcomere striations. In contrast, dead cells were rounded and nonstriated. On reexposure to modified Tyrode solution that contained 1.8 mM $CaCl_2$, the percentage of surviving cells decreased to 40–50%. Electrophysiology was performed on cardiomyocytes that were relaxed, rod-shaped, and distinctly striated.

Electrophysiology

Patch microelectrodes were prepared from filamented glass tubing (1.5 mm outer diameter [OD], borosilicate, Sutter Instruments Co., Novato, CA). Tips were heat polished and filled with a solution that contained (mM) 125 cesium aspartate, 25 tetraethylammonium chloride (TEA), 1 $MgCl_2$, 5 HEPES, 10 EGTA, and 5 MgATP. The pH was adjusted to 7.30 with CsOH. The Cs^+ and TEA minimized potassium currents. Cardiomyocytes were bathed in modified Tyrode solution with 1.8 mM $CaCl_2$ added. In experiments that used Ba^{2+} as the charge carrier, 1.8 mM $BaCl_2$ replaced $CaCl_2$. The Tyrode solution was maintained at 34.7–35.3°C in a 150-μl volume chamber (PDMI-2 Open Perfusion Micro-Incubator, Medical Systems Corp., Greenvale, NY) that was mounted on the stage of an inverted microscope (Diaphot, Nikon Inc., Melville, NY). The superfusion of Tyrode solution, at a rate of 4 ml/min, was continuous throughout the experiment. Electrode tip resistances ranged from 4.2–8.1 MΩ. Tip potentials were adjusted to 0 mV before high-resistance seals were established with the sarcolemma of myocytes (1–10 GΩ). Rupturing the patched membrane with further suction established the whole-cell recording. Normally, the holding potential was set to -40 mV. After a minimum of 10 min to allow the holding current to stabilize between -0.1 and -0.15 nA, $I_{Ca,L}$ was evoked with positive potential command steps. The holding potential of -40 mV was sufficient to fully inactivate voltage-sensitive Na channels and to eliminate current through T-type Ca channels, which were not studied. In some experiments, the holding potential was set to -50 or -20 mV. When the holding potential was -50 mV, 10 μM tetrodotoxin (TTX) was added to the bath, a concentration that proved sufficient to fully block I_{Na}. This was tested by adding $CdCl_2$ (0.2 mM) to the bath. Cd^{2+}, which specifically inhibits $I_{Ca,L}$, completely blocked all inward currents, thereby showing that TTX inhibited I_{Na}. The holding potentials and command potential amplitudes, durations, and frequencies were determined by Clampex software (pClamp6, Axon Instruments, Inc., Foster City, CA), which was run on a Gateway 2000 computer. The computer governed the Axopatch 200A amplifier (Axon Instruments, Inc.) that was outfitted with a CV201A headstage. Current signals, partially corrected for series resistance and whole-cell capacitance, were filtered online at 5 kHz (-3 dB) and stored on hard disk for later analysis by Clampfit software (pClamp6, Axon Instruments, Inc.). The amplitude of $I_{Ca,L}$ was measured from 0 nA. However, in experiments designed to determine current *versus* voltage and steady-state inactivation, the current amplitude was measured as the difference between the peak inward current and the steady-state current near the end of the 200-ms current signal. Bupivacaine HCl (Sigma Chemical Co.) was prepared to varying concentrations in modified Tyrode solution and was administered by superfusion.

To determine whether ATP in the dialyzing pipette solution altered the sensitivity of myocytes to bupivacaine, the response of $I_{Ca,L}$ to 30 μM bupivacaine was measured in the absence of ATP. The presence or absence of ATP had no effect.

Rundown, or the spontaneous decrease in $I_{Ca,L}$ amplitude, was linear with time and did not exceed 0.012 nA/min in bupivacaine-free conditions with ATP in the pipette solution. The formula used to correct for rundown was:

$I_{corr.} = (m \cdot t) + I_t$, where $I_{corr.}$ = peak $I_{Ca,L}$ corrected for rundown (nA), m = rundown slope ($nA \cdot s^{-1}$), t = elapsed time (s), and I_t = peak $I_{Ca,L}$ at time t (nA).

Statistical Analysis

Curves were fit to plotted data using the Marquardt-Levenberg algorithm available in SigmaPlot (Jandel Corp.). Coefficients of determination (R^2) appraised the correctness of fits. Student's *t* test for paired samples was used to compare control *versus* bupivacaine when one concentration of bupivacaine was applied to the myocyte (*e.g.*, concentration-response data). Where noted, Student's *t* test for independent samples was used. When a myocyte was exposed to two concentrations of bupivacaine and washout, multiple pairwise comparisons of data were done with Bonferroni's method. Statistical significance was assumed at $P < 0.05$. Data are presented as mean ± SEM.

Results

Concentration-Response

The peak amplitude of $I_{Ca,L}$ was decreased by bupivacaine (figs. 1, 2). The extent of inhibition that was achieved by each concentration appeared to stabilize by 100 s of application (fig. 2). A wash effect from 10 μM was not seen. Two of the six cells studied in the wash from 10 μM exhibited an inexplicable rapid decay in $I_{Ca,L}$ without a concomitant change in holding current; the decay, therefore, did not appear to be the result of a loss of recording integrity (*e.g.*, seal resistance). The rapid wash-related decay of $I_{Ca,L}$ was not seen for any of the other 16 cells during wash from higher concentrations. Wash only partly, but rapidly, restored the control amplitudes of $I_{Ca,L}$ in 30, 100, and 300 μM. Wash from these higher concentrations restored $I_{Ca,L}$ only to the levels of inhibition approximating that seen in 10 μM of bupivacaine. In figure 2, the rates of inhibition and washout appeared to accelerate with increasing concentrations of bupivacaine. Single exponential fits of the average data ($R^2 > 0.99$) confirmed this impression. Time constants of inhibition were 47, 46, 21, and 15 s for 10, 30, 100, and 300 μM, respectively. Time constants of washout were 48, 49, and 24 s for 30, 100, and 300 μM, respectively. A concentration-response curve (fig. 3) was constructed from the data of figure 2 and was used to estimate the concentration that evoked 50% inhibition (IC_{50}). The average value of IC_{50} was 112.0 ± 9.1 μM.

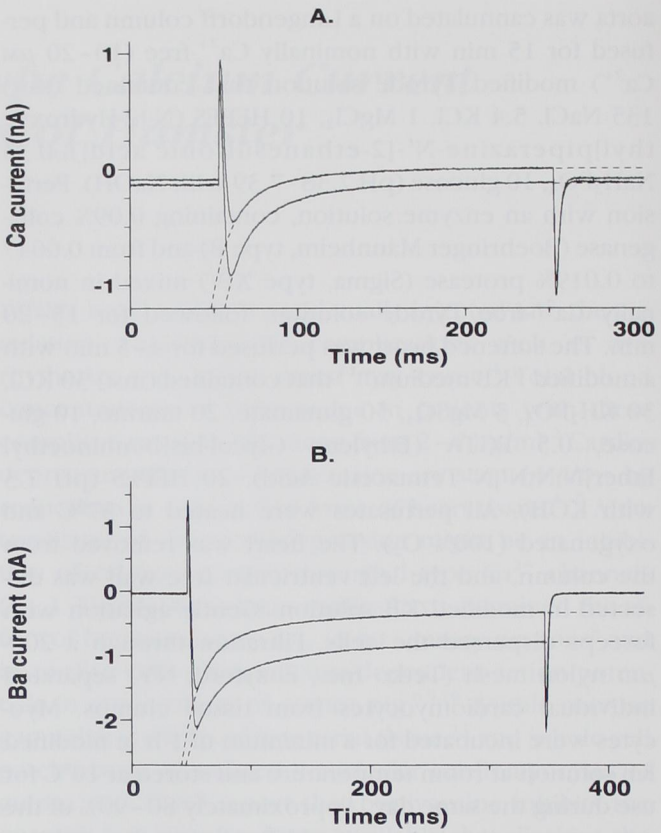

Fig. 1 Representative current traces with (*A*) Ca^{2+}, and (*B*) Ba^{2+} as charge carriers. Currents were evoked by 200 ms (*A*) and 300 ms (*B*) command steps to +5 mV (0.2 Hz stimulus frequency) from a holding potential of -40 mV. *Dashed lines* are fits of the data for determining the two time constants of inactivation by the Simplex least squares fitting method available in Clampfit (tables 1, 2). In (*A*), the two traces show the control and response to 100 μM bupivacaine (2 min) from the same myocyte. Bupivacaine brought the holding current closer to 0 nA. In (*B*), the two traces of $I_{Ba,L}$ show the control and response to 30 μM bupivacaine (2 min) from the same myocyte.

Current-Voltage Relationship

The relationship of $I_{Ca,L}$ to the amplitude of stimulus command voltage (I *vs.* V) is shown in figure 4. Bupivacaine (10 and 100 μM) significantly decreased the amplitude of $I_{Ca,L}$, but did not alter the overall shape of the I *versus* V relationship, except at -30 and -20 mV, wherein the activation component appeared to be depressed by bupivacaine and could not be reversed with wash.

Time to Activation and Rates of Inactivation

The time to activation of $I_{Ca,L}$ was appraised by the time between the start of stimulation and the current

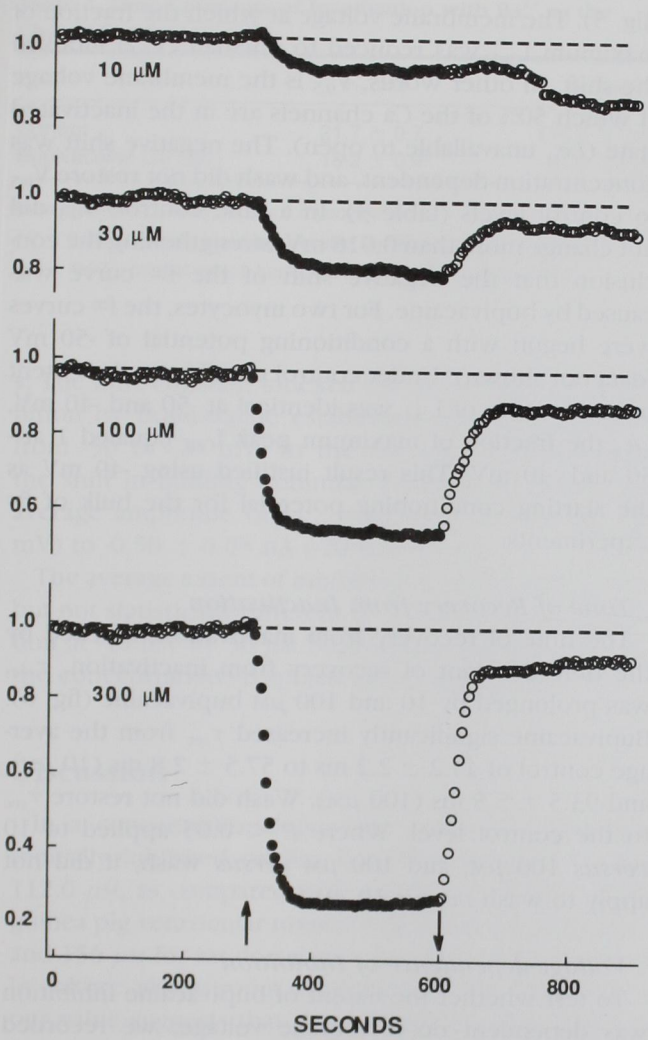

Fig. 2. Inhibition of $I_{Ca,L}$ by four concentrations of bupivacaine. Ordinates are fractions of peak $I_{Ca,L}$ (*i.e.*, $I_{Ca,I}/I_{Ca,L}$ control) after correction for rundown, which did not exceed 0.012 nA/min. Currents were evoked as in figure 1A. Five minutes of control (*open circles*) was followed by 5 min of bupivacaine (*filled circles*, onset at *upward arrow*), and 5 min of wash (*open circles*, onset at *downward arrow*). N = 6 (10 μM), 5 (30 μM), 5 (100 μM), and 6 (300 μM) myocytes. SEM bars were not shown (see fig. 3 for SEM bars).

peak (time-to-peak [TTP]) from the same cells used in the concentration-response study (figs. 1A, 2, and 3). TTP increased slightly, but significantly, in all concentrations of bupivacaine (10, 30, 100, and 300 μM). Because the increase was dose-independent ($P > 0.19$ by Student's t test for independent samples), we report only the average TTP for control and bupivacaine records. These were 7.8 ± 0.2 ms (n = 22) for control and 9.0 ± 0.3 ms (n = 22) for bupivacaine ($P < 0.001$). The average increase in TTP was 1.1 ± 0.2 ms above the control value. Wash (5 min) did not restore TTP to the control level. When Ba^{2+} was the charge carrier, the increase in TTP in 30 μM bupivacaine was not statistically significant from control ($P > 0.10$). The values of TTP were 8.5 ± 0.4 ms (n = 5) for control and 9.3 ± 0.6 ms for 30 μM bupivacaine. Also, there was no significant difference between the control and wash averages of TTP. The durations of capacitative transients were from 1.44 to 3.12 ms and for each cell did not change by more than 0.36 ms during an experiment (control-bupivacaine-washout). Therefore, it is likely that measurements of TTP were not significantly distorted by the capacitative transients that persisted after partial correction.

Inactivation, or the spontaneous decay of $I_{Ca,L}$, was fit to a biexponential model to extract two time constants of inactivation (τ_{f1} and τ_{f2}). These are reported in table 1 for those cells used in the concentration-response study (figs. 1A, 2, and 3). There was a slight, but significant, decrease in τ_{f1} in 30 μM, and τ_{f2} significantly in-

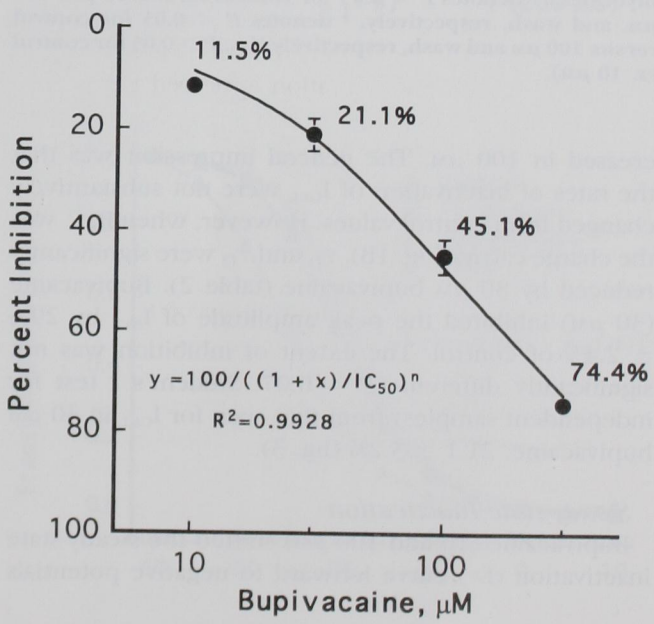

Fig. 3. Inhibition of peak $I_{Ca,L}$ *versus* bupivacaine concentration. Data are measured from the same cells used in figure 2, and peak currents were measured at 5 min of drug application. Extents of inhibition (Δ% of control) are given. The data points were fit (*solid lines*) to a logistic function (*inset*) for estimating IC_{50}, where y and x are ordinate and abscissa values, respectively. IC_{50} = 112.0 ± 9.1 μM, and the slope factor n = -0.9881 ± 0.008 μM. The *filled circles* are larger than the SEM bars for 10 and 300 μM. The size of SEM bars are the same as for the data point averages of figure 2.

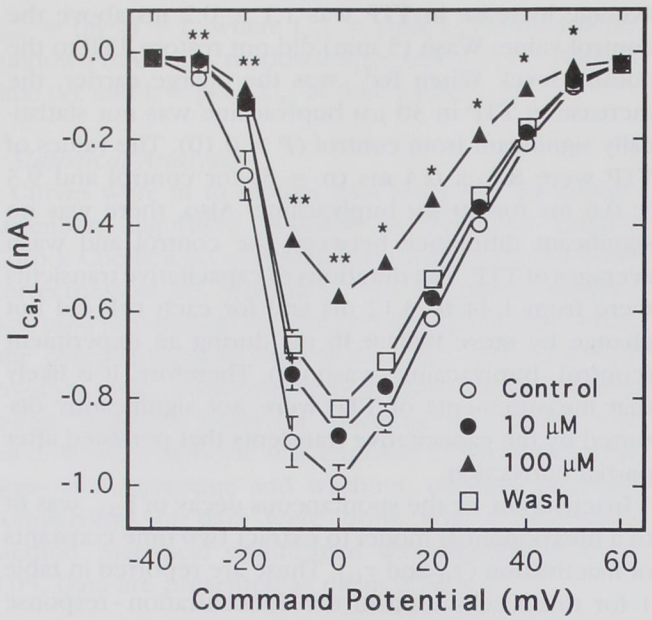

Fig. 4. Current *versus* voltage relationship. Averages of peak amplitudes of $I_{Ca,L}$ were plotted against command voltages. Records were obtained at 2 to 2.5 min of exposure to each concentration, or 2 to 2.5 min of wash. Data are from five myocytes. ** denotes $P < 0.05$ for control *versus* 10 μM, 100 μM, and wash, respectively. * denotes $P < 0.05$ for control *versus* 100 μM and wash, respectively (*i.e.*, $P > 0.05$ for control *vs.* 10 μM).

creased in 100 μM. The general impression was that the rates of inactivation of $I_{Ca,L}$ were not substantively changed from control values. However, when Ba^{2+} was the charge carrier (fig. 1B), τ_{f1} and τ_{f2} were significantly reduced by 30 μM bupivacaine (table 2). Bupivacaine (30 μM) inhibited the peak amplitude of $I_{Ba,L}$ by 29.6 ± 2.4% of control. The extent of inhibition was not significantly different ($P = 0.07$; Student's *t* test for independent samples) from that seen for $I_{Ca,L}$ in 30 μM bupivacaine, 21.1 ± 3.2% (fig. 3).

Steady-state Inactivation

Bupivacaine (10 and 100 μM) shifted the steady-state inactivation (f_∞) curve leftward to negative potentials (fig. 5). The membrane voltage at which the fraction of maximum $I_{Ca,L}$ was reduced to one-half ($V_{0.5}$) indexed the shift. In other words, $V_{0.5}$ is the membrane voltage at which 50% of the Ca channels are in the inactivated state (*i.e.*, unavailable to open). The negative shift was concentration-dependent, and wash did not restore $V_{0.5}$ to control levels (table 3). In a time control, $V_{0.5}$ did not change more than 0.018 mV, strengthening the conclusion that the negative shift of the f_∞ curve was caused by bupivacaine. For two myocytes, the f_∞ curves were begun with a conditioning potential of -50 mV (data not shown). Under control conditions, the extent of inactivation of $I_{Ca,L}$ was identical at -50 and -40 mV, *i.e.*, the fraction of maximum peak $I_{Ca,L}$ equaled 1 at -50 and -40 mV. This result justified using -40 mV as the starting conditioning potential for the bulk of f_∞ experiments.

Time of Recovery from Inactivation

The time of recovery from inactivation, indexed by the time constant of recovery from inactivation, τ_{rec}, was prolonged by 10 and 100 μM bupivacaine (fig. 6). Bupivacaine significantly increased τ_{rec} from the average control of 44.2 ± 2.2 ms to 57.5 ± 2.8 ms (10 μM), and 93.5 ± 5.5 ms (100 μM). Wash did not restore τ_{rec} to the control level. Where $P < 0.05$ applied to 10 *versus* 100 μM, and 100 μM *versus* wash, it did not apply to wash *versus* 10 μM.

Voltage-dependence of Inhibition

To test whether the extent of bupivacaine inhibition was dependent on membrane voltage, we recorded peak amplitudes of $I_{Ca,L}$ at holding potentials of -50 and -20 mV. The data are presented in figure 7. At -50 mV, bupivacaine (30 μM) inhibited $I_{Ca,L}$ by 30.2 ± 4.1% (Δ% of control). The change to -20 mV increased the inhibition by 55.8 ± 6.0% ($P < 0.008$ for -50 *vs.* -20 mV data). Note that the amplitude of $I_{Ca,L}$ was stable by 6–8 s. A monoexponential function was fit to the -20 mV data between 2–10 s with R^2 values between from 0.977 to 0.996. The average time constant equalled 1.40 ± 0.08

Table 1. Time Constants of Inactivation in Different Concentrations of Bupivacaine HCl

	Control	*versus*	10 μM	Control	*versus*	30 μM	Control	*versus*	100 μM
τ_{f1} (ms)	14.0 ± 1.1		14.3 ± 1.0	14.8 ± 1.1		13.1 ± 0.8*	10.7 ± 0.9		10.8 ± 0.4
τ_{f2} (ms)	44.9 ± 4.4		47.8 ± 4.5	50.5 ± 7.8		45.4 ± 4.1	35.7 ± 3.0		41.0 ± 1.8*

Data are mean ± SEM for n = 5–6 myocytes. Data are from the concentration–response analysis (see fig. 2).

* $P < 0.05$, control *versus* bupivacaine by Student's paired *t* test.

Table 2. Time Constants of Inactivation with Ba^{2+} as the Charge Carrier

	τ_{f1} (ms)	τ_{f2} (ms)
Control	21.7 ± 0.7	115.0 ± 7.4
Bupivacaine (30 μM)	18.2 ± 0.9*	97.9 ± 5.4†

Data are mean ± SEM for n = 5 myocytes.

* $P < 0.002$, control *versus* bupivacaine by Student's paired *t* test.

† $P < 0.025$, control *versus* bupivacaine by Student's paired *t* test.

s. The time constant indexed the rate of increased inhibition by bupivacaine established after the transition from -50 to -20 mV. In the absence of bupivacaine, the shift in holding potential, by itself, decreased the average amplitude of $I_{Ca,L}$ from -1.05 ± 0.05 nA (-50 mV) to -0.50 ± 0.05 nA (-20 mV) by 20 s.

The average extent of inhibition at -50 mV was greater but not statistically different ($P = 0.15$) than the inhibition at -40 mV by 30 μM bupivacaine that was seen for the concentration–response plot of figure 3.

Discussion

In a concentration-dependent manner, bupivacaine tonically inhibited cardiac $I_{Ca,L}$. The average IC_{50} was 112.0 μM, as compared with an estimated 80.1 μM for guinea pig ventricular myocytes at room temperature,[12] and 156 μM for rat dorsal root ganglion (DRG) neurons in culture, also at room temperature.[8] The mid-range of our value suggests that temperature has little effect in altering the sensitivity of Ca channels to bupivacaine. The local anesthetic might be acting to increase the probability that L-type Ca channels reside in the inactivated state. The evidence for this comes from the negative-voltage shift in the steady-state inactivation curve (f_∞) and the increase in time of recovery from inactivation (τ_{rec}). The former suggests that fewer channels are available to open, whereas the latter indicates that it takes longer for the inactivated channels to recover before they become available to open. It is possible that tonic and use-dependent (phasic) inhibition may be occurring together in the normally functioning heart because bupivacaine may extend the time of recovery from inactivation to a point that the diastolic duration is too short to allow for a complete recovery from inactivation. However, bupivacaine is reported not to have a significant use-dependent effect.[12] It is also reported that the f_∞ curve is not shifted by 50 μM bupivacaine in ventricular cardiomyocytes of guinea pig,[12] a discrepancy which we are unable to explain.

In the present study, the Ca^{2+} chelator, EGTA, in the pipette dialyzing solution reduced but did not eliminate the effect of Ca-dependent inactivation on $I_{Ca,L}$.[15] The decreased influx of Ca^{2+} caused by bupivacaine might also have increased the inactivation time constants and masked an accelerating effect on the rates of inactivation. This idea is supported by the report that in frog atrial fibers, bupivacaine increased the inactivation time constant using a voltage clamp technique that did not buffer intracellular Ca^{2+}.[11] The possibility of bupivacaine accelerating inactivation was tested by using Ba^{2+} as the charge carrier, thereby eliminating Ca-dependent

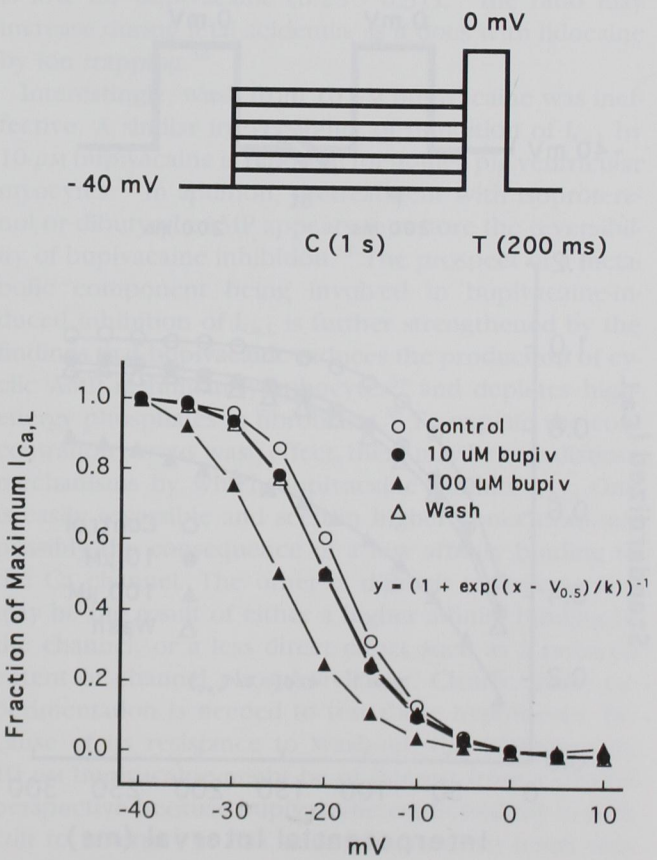

Fig. 5. Steady-state inactivation (f_∞). The schematic depicts the stimulus protocol, which was repeated every 5 s. Variable amplitude conditioning potentials (C) were followed by a test potential (T). Amplitudes of C (shown only to -10 mV) were extended to +10 mV. As C was increased, the amplitude of $I_{Ca,L}$ evoked by T decreased. The fraction of maximum $I_{Ca,L}$ evoked by T was plotted against the amplitude of C. Estimates of $V_{0.5}$ and slope factor (k) were obtained using the Boltzmann function (*inset*), and are given in table 3. Data are from six myocytes. R^2 for fits were not less than 0.998.

Table 3. Parameters Derived from Fits of Steady-State Inactivation Curves

	Control	10 μM	100 μM	Wash
$V_{0.5}$ (mV)	−18.1 ± 0.3	−19.8 ± 0.2*	−24.8 ± 0.1*	−19.7 ± 0.2*
k (mV)	4.0 ± 0.2	4.1 ± 0.1	4.4 ± 0.1	4.3 ± 0.1

Data are mean ± SEM for n = 6 myocytes.

* For $V_{0.5}$, $P < 0.05$ for control *versus* 10 μM, 100 μM, and wash. There were no significant differences between 10 μM and wash, whereas 10 *versus* 100 μM, and 100 μM *versus* wash were significantly different. For k, $P < 0.05$ applied only for control *versus* 100 μM, and 10 *versus* 100 μM.

inactivation as a factor. Under this condition, bupivacaine accelerated inactivation, although its contribution to decreasing the amplitude of $I_{Ca,L}$ when Ca- and voltage-dependent inactivation are operative remains uncertain. The time to channel opening was slightly increased by bupivacaine, as suggested by the increase in TTP current. A bupivacaine-induced increase of TTP for $I_{Ca,L}$ also is reported in cultured sensory neurons of rat[8] and in frog atrial fibers.[11]

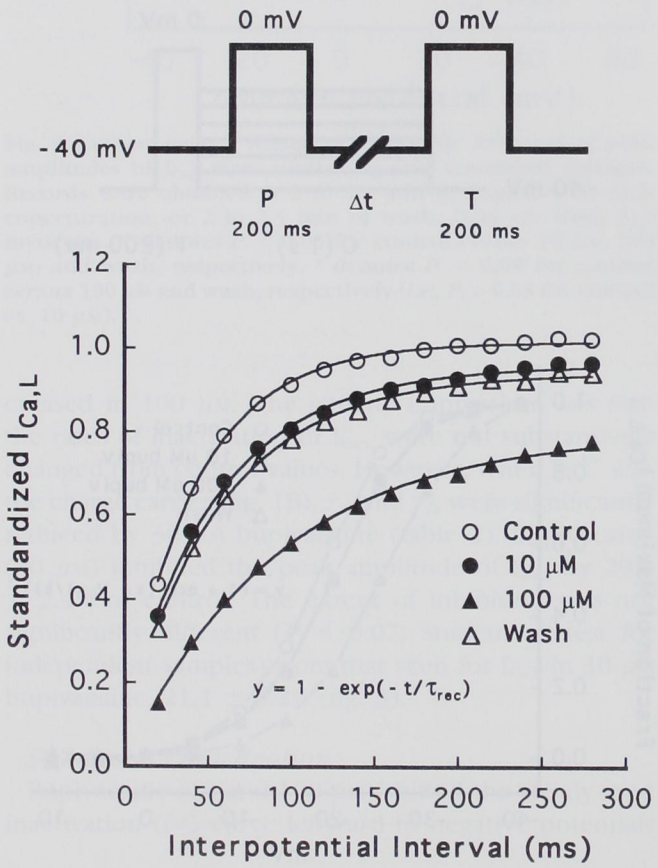

Fig. 6. Time of recovery from inactivation. Data points are the means of six myocytes. The schematic summarizes the stimulus protocol, which was repeated every 5 s. A test potential (T) followed a prepotential (P) by increasing intervals of time (Δt). Standardized $I_{Ca,L}$ (peak amplitude ratio of $I_{Ca,L}$, T/P) was plotted against the interpotential interval (Δt). The data were fit to a monoexponential function (*inset*), where t = abscissa values, to yield the solid line curves and estimates of the time constant of recovery from inactivation, τ_{rec}. R^2 for fits were not less than 0.997.

If a voltage-dependence to the inhibition of $I_{Ca,L}$ by bupivacaine could be shown, an explanation of the mechanism of inhibition would be consistent with the modulated receptor hypothesis.[16,17] Accordingly, bupivacaine would bind to channels differently depending on whether they were open, closed, or inactivated. If bupivacaine predisposes Ca channels to the inactivated state, it might be because it binds with greater affinity to the inactivated channels. For example, a greater proportion of Ca channels would be expected to be in the inactivated state at -20 mV (40% inactivated by fig. 5) than at a more hyperpolarized value, and our results showed that bupivacaine inhibited $I_{Ca,L}$ more at -20 than -50 mV. Two studies that used ventricular cardiomyocytes of guinea pig report different results in this regard. One shows no difference in the extent of tonic inhibition at -40 and -70 mV holding potentials,[12] whereas the other demonstrates much more inhibition at -40 mV than at -90 mV.[9]

The characteristics of inhibition of $I_{Ca,L}$ by bupivacaine have similarities and differences with that by dihydropyridine antagonists. Nisoldipine and nitrendipine, for example, prolong the time of recovery from inactivation of $I_{Ca,L}$.[18,19] Nevertheless, use-dependency of dihydropyridine block is much milder than that of diarylalkylamines (verapamil and D600).[20,21] The voltage-dependency of Ca channel block by dihydropyridines is marked compared with bupivacaine, and there is a rapid and complete washout of nitrendipine.[22] Nisoldipine causes a hyperpolarizing shift in the steady-state inactivation curve of $I_{Ca,L}$,[19] suggesting strongly that dihydropyridines interact with the inactivated state of the Ca channel. Finally, inhibition of cardiac $I_{Ca,L}$ by bupivacaine is more potent in the presence of nifedipine, although it is uncertain which drug potentiates the other.[9]

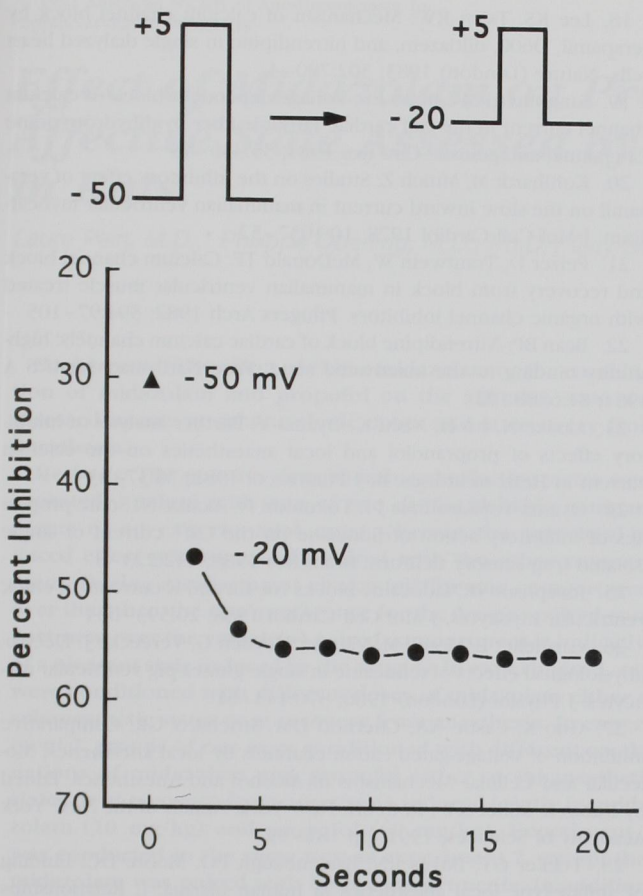

Fig. 7. Voltage-dependence of bupivacaine inhibition (30 μM). $I_{Ca,L}$ was evoked by 200 ms command steps to +5 mV from a holding potential of -50 mV (0.5 Hz) (*solid triangle*). After the peak amplitude of $I_{Ca,L}$ was stable, the holding potential was abruptly changed to -20 mV and 200 ms command steps to +5 mV were continued at 0.5 Hz (*solid circles*). $I_{Ca,L}$ was recorded in the presence of 10 μM TTX, which was sufficient to eliminate I_{Na} during the step from -50 to +5 mV. Data are the average of three cardiomyocytes.

Other local anesthetics inhibit $I_{Ca,L}$ in neurons[8,23,24] and ventricular cardiomyocytes of chicks[25] and guinea pigs.[26] Tetracaine decreases the time constant of the slowly inactivating component of $I_{Ca,L}$ in ventricular myocytes of guinea pig when Sr^{2+} or Ba^{2+} is the charge carrier.[26] In a comparison of the inhibitory effects of local anesthetics on $I_{Ca,L}$ of rat DRG neurons, potencies are ranked as dibucaine > tetracaine > bupivacaine >> procaine = lidocaine; a sequence, the authors note, that closely matches the respective lipid solubilities.[8] In addition, other types of Ca channels have been examined for their sensitivity to local anesthetics. Tetracaine inhibits I_{Ca} in N- and T-type Ca channels of rat DRG neurons.[8] Also, N-type I_{Ca} in frog DRG neurons is inhibited by a derivative of bupivacaine, HS37, the S(-) being more potent than the R(+) isomer.[27]

The lowest concentration of bupivacaine HCl used in this study, 10 μM (3.25 mg/ml), appears to be of dubious clinical significance because bupivacaine is reported to be 95.6% (0.4 mg/ml) to 87.7% (5.0 mg/ml) bound to human plasma protein.[28] However, 3.25 mg/ml may approach clinical relevance when free bupivacaine is increased by other drugs that displace it from plasma protein[29] or when bupivacaine potency is increased by combining it with calcium antagonists.[5,9] Also, fetuses may be more susceptible to bupivacaine because the percent of bound bupivacaine in fetal blood averages 66% at 1 mg/ml and 51% at 5 mg/ml.[30] Although the umbilical vein to maternal arterial concentration ratio is low for bupivacaine (0.25 - 0.31),[31] the ratio may increase during fetal acidemia, as it does with lidocaine by ion trapping.[32]

Interestingly, wash from 10 μM bupivacaine was ineffective. A similar irreversibility of inhibition of $I_{Ca,L}$ by 10 μM bupivacaine is reported for guinea pig ventricular myocytes.[33] In addition, pretreatment with isoproterenol or dibutyryl cAMP appears to restore the reversibility of bupivacaine inhibition.[33] The prospect of a metabolic component being involved in bupivacaine-induced inhibition of $I_{Ca,L}$ is further strengthened by the findings that bupivacaine reduces the production of cyclic AMP in human lymphocytes[34] and depletes high-energy phosphates in fibroblasts.[35] To explain the concentration *versus* wash effect, there may be two distinct mechanisms by which bupivacaine inhibits $I_{Ca,L}$. One is easily reversible and seen in higher concentrations, possibly the consequence of a low affinity binding to the Ca channel. The other is difficult to reverse and may be the result of either a higher affinity binding to the channel, or a less direct effect such as a reduced extent of channel phosphorylation. Clearly, more experimentation is needed to test these hypotheses. Because of its resistance to washout, the inhibition by 10 μM bupivacaine might be of interest from a clinical perspective because bupivacaine cardiotoxicity is difficult to reverse.[1] In this sense, the *in vitro* result may be mimicking what is seen clinically.

In summary, bupivacaine tonically inhibits $I_{Ca,L}$ in hamster ventricular cardiomyocytes. The changes in steady-state inactivation and time course of recovery from inactivation suggest that the local anesthetic predisposes L-type Ca channels to the inactivated state. Because wash from the low concentration is ineffective, a second mechanism of inhibition may be involved.

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