

Effect of Local Anesthetic on Neuronal Cytoplasmic Calcium and Plasma Membrane Lysis (Necrosis) in a Cell Culture Model

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Background: To investigate the mechanism by which rare cases of spinal local anesthetic (LA) neurotoxicity occur, we have tested the hypotheses that LAs elevate cytoplasmic calcium ($\text{Ca}^{2+}_{\text{cyt}}$), that this is associated with a neurotoxic effect, and that lidocaine and bupivacaine differ in their neurotoxicity.

Methods: Neurons of the ND7 cell culture line, derived from dorsal root ganglion, were loaded with fura-2 and analyzed by digitized video fluorescence microscopy during 60 min LA exposure, allowing determination of $\text{Ca}^{2+}_{\text{cyt}}$ and time of necrotic cell death (plasma membrane lysis) at the single neuron level.

Results: Lidocaine 0.1% and bupivacaine 0.025% caused minimal changes in $\text{Ca}^{2+}_{\text{cyt}}$. Lidocaine 0.5–5% and bupivacaine 0.125–0.625% caused an early, small (less than threefold), concentration-dependent increase in $\text{Ca}^{2+}_{\text{cyt}}$ that was transient and returned to near baseline within 10 min. Lidocaine 2.5% and 5% then caused a sustained, greater than ten-fold increase in $\text{Ca}^{2+}_{\text{cyt}}$ and death in some neurons during the 60 min exposure period. Pretreatment with thapsigargin eliminated the initial transient increase in $\text{Ca}^{2+}_{\text{cyt}}$, consistent with endoplasmic reticulum (ER) as its source, and increased neuronal death with 5% lidocaine, suggesting that lidocaine neurotoxicity can be increased by failure of ER to take up elevated $\text{Ca}^{2+}_{\text{cyt}}$. The later sustained increase in $\text{Ca}^{2+}_{\text{cyt}}$ seen with 2.5 and 5% lidocaine was prevented in Ca^{2+} -free medium, and restored when Ca^{2+} was added back to the buffer in the presence of lidocaine, suggesting that higher concentrations of lidocaine increase influx of Ca^{2+} through the plasma membrane.

Conclusions: In this model, lidocaine greater than 2.5% elevates $\text{Ca}^{2+}_{\text{cyt}}$ to toxic levels. Bupivacaine and lower concentrations of lidocaine transiently alter $\text{Ca}^{2+}_{\text{cyt}}$ homeostasis for several minutes, but without an immediate neurotoxic effect within 60 min.

CLINICAL,^{1,2} *in vivo*,³ and *in vitro*⁴ studies have documented the occurrence of local anesthetic (LA) spinal neurotoxicity, dependent on both concentration and

exposure time, but not determined the mechanism by which LAs cause neurotoxicity. It is difficult to postulate a mechanism of neural damage based on the primary pharmacologic effect of LAs, block of Na^+ channels. Blockade of Na^+ channels and resultant electrical inactivity should decrease neuronal metabolism and preserve ATP. Since export of $\text{Ca}^{2+}_{\text{cyt}}$ to the extracellular space is coupled to influx of Na^+ through the Na^+ - Ca^{2+} exchanger, block of Na^+ channels and maintenance of a low cytosolic Na^+ should act to prevent elevation of $\text{Ca}^{2+}_{\text{cyt}}$.⁵ Furthermore, LAs not only block Na^+ channels, but also block Ca^{2+} channels at higher concentrations.⁶ LAs (at concentrations near therapeutic plasma levels, but much lower than CSF concentrations during spinal anesthesia) limit the increase in $\text{Ca}^{2+}_{\text{cyt}}$ caused by agents which cause an influx of Ca^{2+} through the plasma membrane, in myocardium,⁷ airway smooth muscle,⁸ and secretory cells.⁹ Blockade of Na^+ influx is protective during neuronal anoxia.¹⁰ LAs are neurotoxic and the chemically dissimilar tetrodotoxin is not when both are given as spinal anesthetics to rats at concentrations that produce similar extents of Na^+ block.³

It is therefore most likely that a neurotoxic effect of LAs is mediated by effects other than Na^+ channel blockade. One potential mechanism is prolonged elevation of $\text{Ca}^{2+}_{\text{cyt}}$, in contrast to the physiologic, fleeting elevation of $\text{Ca}^{2+}_{\text{cyt}}$, which occurs during response to neurotransmitters.^{5,11-13} Previous studies have suggested a possible detrimental effect of LAs on Ca^{2+} release from nonmitochondrial intracellular stores.¹⁴⁻¹⁷ Those studies were limited by being performed on subcellular fragments, rather than on an intact cell with a native intracellular environment. In a glial cell line, lidocaine caused an increase in $\text{Ca}^{2+}_{\text{cyt}}$ in cells surrounded by Ca^{2+} -free buffer, implying release from an intracellular store.¹⁸ In adult dorsal root ganglion neurons, a 30 s pulse of lidocaine transiently increased $\text{Ca}^{2+}_{\text{cyt}}$, deriving from both intracellular and extracellular Ca^{2+} stores.¹⁹

In the studies reported here, we tested the hypotheses that LAs alter neuronal Ca^{2+} homeostasis and cause a sustained increase in $\text{Ca}^{2+}_{\text{cyt}}$, that this is associated with a neurotoxic effect, and that lidocaine and bupivacaine differ in their neurotoxicity. We have utilized a cell culture line of healthy sensory neurons to allow assays at the single neuron level, to minimize effects of preparative trauma on $\text{Ca}^{2+}_{\text{cyt}}$ and neuronal injury, and to eliminate vascular and other systemic effects of LA. In this model, we have determined the response of neuronal

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$\text{Ca}^{2+}_{\text{cyt}}$ to lidocaine and bupivacaine during a 60 min exposure to clinically relevant concentrations, ranging from that expected with maldistribution and minimal mixing of high concentrations with CSF, to that expected with complete mixing of lower concentrations. We have also determined the incidence of neuronal death during the 60 min exposure.

Materials and Methods

Chemicals and Buffers

All drugs and other chemicals were obtained from Sigma-Aldrich (St. Louis, Missouri), except where specifically noted, and were of the highest purity available. LAs were obtained as their hydrochloride salts, dissolved in buffer, and adjusted to pH 7.4 prior to use. Expressions of percent LA (g/dl) were calculated from the hydrochloride salt weight, consistent with standard clinical usage. Experimental buffer was HEPES-buffered KRH: 5 mM D-glucose; 25 mM HEPES; 115 mM NaCl; 5 mM KCl; 1.2 mM MgSO_4 ; 1.0 mM KH_2PO_4 ; 2.0 mM CaCl_2 ; +NaOH to pH 7.4.¹³

Cell Culture

All neuronal studies were conducted using the ND7-104 subclone of the ND7 cell line, obtained from Patrick G. Hogan, Ph.D., Investigator, The Center for Blood Research, Boston, Massachusetts. ND7 was derived from rat dorsal root ganglion, immortalized by fusion with mouse neuroblastoma, and has been extensively characterized as a sensory neuron model.²⁰⁻²³

Neuronal cultures were started with an aliquot grown from the original ND7-104 subclone stock, and used for the lesser of 2 months or 8 passages. Proliferation medium for routine cell growth was L-15 (Gibco BRL Life Technologies, Grand Island, New York) supplemented with 3.3 g/l NaHCO_3 , 3.3 g/l d-glucose, and 10% fetal calf serum (FCS; Hyclone; Logan, Utah), in T75 flasks. Neuronal suspensions were prepared by gentle trypsinization (0.05% trypsin + 0.53 mM EDTA) for 3 min, followed by FCS to inhibit further proteolysis, centrifugation at $228 \times g$ for 5 min (Beckman GPR centrifuge; Fullerton, California), resuspension in proliferation medium, and plating on glass coverslips.

Round glass coverslips (Fisherbrand 25CIR-1; Fisher Scientific, Pittsburgh, Pennsylvania) were dipped in 75% ethanol and put with sterile forceps into Falcon tissue culture 35 \times 10 mm dishes (BD Biosciences, Franklin Lakes, New Jersey), then exposed to ultraviolet light for 20 min. Poly-D-lysine hydrobromide (Sigma P7405, molecular weight > 300,000; Sigma-Aldrich, St. Louis, Missouri) 0.1 mg/ml dH_2O was added, 1.5 ml per dish, and incubated 18 h at room temperature. Each dish was then washed twice with 2 ml sterile dH_2O , then 1 ml proliferation medium added and the dish and coverslip incubated at 37°C for 30 min. Neurons were then added as a suspension to the coverslip in the tissue culture dish.

Neurons were allowed to attach for 6 h, then the medium was changed to differentiation medium, which is identical to proliferation medium except that FCS was decreased to 0.5%, and 1 mM cyclic adenosine monophosphate (cAMP) and 2 ng/ml nerve growth factor (recombinant rat NGF- β) were added. This medium was changed daily, and the cells used 48-72 h after they were initially exposed to differentiation medium. Cells were incubated at 37°C in 6% CO_2 , remainder room air.

Digitized Video Fluorescence Microscopy

We have previously described^{13,24,25} the imaging system that was used: an Attofluor RatioVision system (Atto Instruments, Rockville, MD) using a Zeiss (Carl Zeiss MicroImaging, Inc., Thornwood, New York) Axiovert 35 M inverted microscope with a Zeiss 40 \times , 1.30 NA, oil, Plan, Neofluar lens, equipped with an ICCD camera and a temperature controlled stage. The vendor's software was used for defining regions of interest (*i.e.*, single cells), background subtraction, pixel by pixel ratioing and calibration, and gray value reporting of the unprocessed image to insure that the 8 bit dynamic range of the video camera was not exceeded. Neurons were completely shielded from the excitation light except during the fraction of a second when an image was acquired. All experiments were conducted at $37.0 \pm 0.3^\circ\text{C}$.

Neurons were loaded with 5 μM fura-2 acetoxymethyl ester (Molecular Probes, Eugene, OR) in KRH + 10% fetal calf serum (FCS) for 20 min at 37°C. This yielded cytoplasmic fura-2: neurons displayed a diffuse, non-punctate fluorescence throughout the neuron, and lost more than 95% of their fluorescence when exposed to 20 μM digitonin, which lyses plasma membrane but not organellar membranes.²⁶ Excitation was at 334 nm, and 380 nm, with 510 nm dichroic, and 540 ± 25 nm emission. Calculation of $\text{Ca}^{2+}_{\text{cyt}}$ from fura-2 fluorescence ratios was performed by calibration with fura-2-free acid solutions containing no Ca^{2+} (10 mM EGTA) and saturating Ca^{2+} (2 mM), in 100 mM KCl, 10 mM NaCl, 10 mM MOPS, pH 7.2.¹³ Neuronal death was detected by the sudden loss of fura-2 fluorescence from the fluorescent image, indicating loss of plasma membrane integrity, with the lysed neuronal membrane remaining in position on phase contrast view. Dead neurons were excluded from $\text{Ca}^{2+}_{\text{cyt}}$ analysis after the time of death. At the time of neuronal death, plasma membrane integrity is lost and there is no longer an effective barrier to the influx of Ca^{2+} along its large concentration gradient from outside the neuron. Apparent $\text{Ca}^{2+}_{\text{cyt}}$ values at this time provide no indication about the role of $\text{Ca}^{2+}_{\text{cyt}}$ in leading to neuronal death, but are predictably high as a result of neuronal death.

Following loading with fura-2, any planned pretreatment, and establishment of a stable pre-LA baseline for microscopy experiments, LA was added to yield the final concentration indicated for a given experiment. Images

were acquired at a fixed interval for the duration of the experiment, which was planned for 60 min for LA exposure without pretreatment. Control experiments with equimolar concentrations of Tris buffer in place of LA were also performed to control for osmotic and other effects. Tris [(tris-hydroxymethyl)aminomethane] is a commonly used laboratory buffer, with a substituted amine pK_a of 8.1 (cf. lidocaine $pK_a = 7.9$, bupivacaine $pK_a = 8.1$).

Statistical Analysis of Changes in Ca^{2+}_{cyt}

Because such calibrated values of Ca^{2+}_{cyt} are of highest accuracy in comparing successive values in the same neuron, rather than comparing absolute values between neurons,²⁷ our experimental protocols were analyzed using the normalized percent change in Ca^{2+}_{cyt} from the single neuron's baseline before comparing it with other neurons' changes. Initial Ca^{2+}_{cyt} was 126 ± 13 (SD) nM; $N = 967$ neurons. Ca^{2+}_{cyt} concentration at each time point following LA addition for each neuron was normalized by dividing by the average Ca^{2+}_{cyt} value for that neuron in the 5 min preceding addition of LA. These normalized values were averaged separately for the periods of 0–10 min, and 10–60 min (time periods based on initial inspection of data; see Results). Each value was weighted in inverse proportion to the number of neurons observed in a given experiment, so that each experiment had equal weight. Statistical analysis was then performed on the normalized, weighted 0–10 min, and 10–60 min, averages of each neuron.

For statistical comparisons between LAs, bupivacaine and lidocaine were compared at equipotent concentrations using a ratio of 1:4; e.g., 0.25% bupivacaine was considered equipotent to, and the same concentration for statistical analysis as, 1% lidocaine. The equimolar Tris buffer controls for bupivacaine and lidocaine were also considered as separate LAs for statistical analysis, and compared with their corresponding equimolar LA concentration; e.g., lidocaine 1% (37 mM) was compared with Tris buffer equimolar (37 mM) to lidocaine 1%.

To determine the effect of concentration, linear regression for each LA was performed separately for 0–10 min, and 10–60 min, and for –Thapsigargin (Tps) and +Tps. To determine the effect of LA, analysis of variance was performed at each LA concentration, using LA and Tps as independent variables, and 0–10 min and 10–60 min Ca^{2+}_{cyt} averages as repeated measures dependent variables. *Post hoc* comparisons within significant effects were performed using the Bonferroni correction.

Survival Analysis

Time of death data from all neurons under a given condition of LA and Tps were combined to produce a single Kaplan-Meier estimator for each condition, and then compared by the Kruskal-Wallis test. All statistical

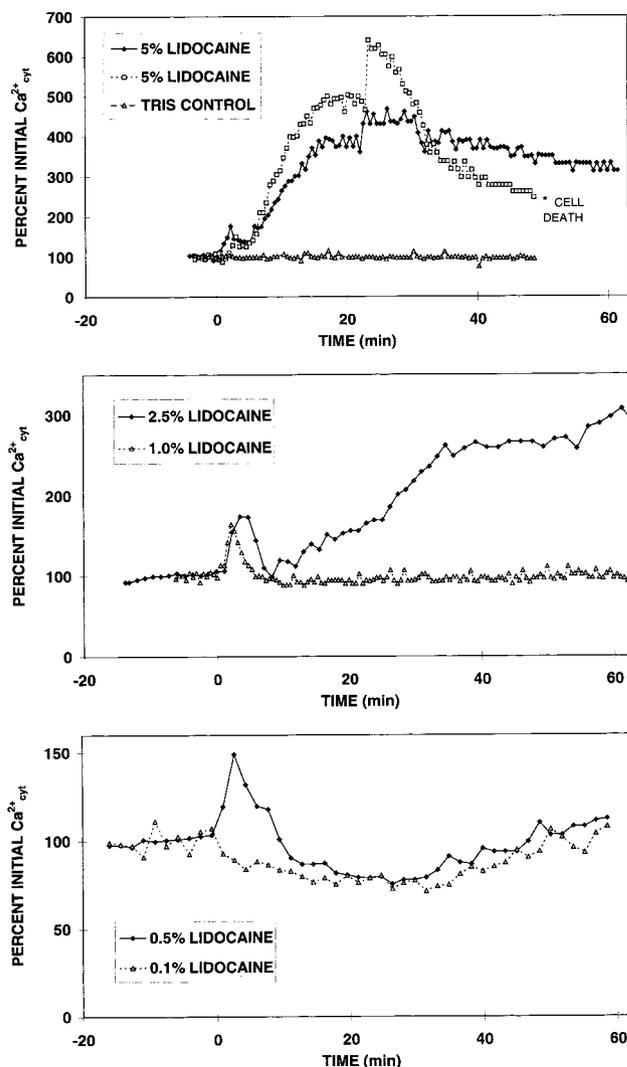


Fig. 1. Effect of lidocaine on neuronal Ca^{2+}_{cyt} : Representative single neuron Ca^{2+}_{cyt} tracings. The asterisk indicates plasma membrane lysis and necrotic cell death for the indicated neuron after exposure to 5% lidocaine, after which Ca^{2+}_{cyt} values are not meaningful because the cytoplasm is in direct communication with extracellular buffer. Note: two-fold expansion of y-axis for each successive graph at lower lidocaine concentrations. Neurons selected for the 5 and 2.5% lidocaine plots represent the lower end of Ca^{2+}_{cyt} values observed for these concentrations, to more clearly illustrate the initial small peak in Ca^{2+}_{cyt} (cf. figs. 3 and 4). The Tris control illustrated was equimolar to 5% lidocaine HCl (i.e., 185 mM). Tris controls for less than 5% lidocaine are omitted for clarity; all were similar to the Tris control illustrated for 5% lidocaine; i.e., flat with minimal increase. Tracings are representative of $N = 5$ to 6 experiments for 1.0–5.0% lidocaine; and of $N = 3$ to 4 experiments for 0.1–0.5% lidocaine and for all Tris controls; number of neurons per experiment = 17 ± 5 (SD).

calculations were performed using the algorithms of Systat 7.01 (SPSS Inc., Chicago, Illinois).

Results

Effect of LA on Neuronal Ca^{2+}_{cyt}

Qualitative Description. Figure 1 illustrates representative single neuron Ca^{2+}_{cyt} responses to lidocaine

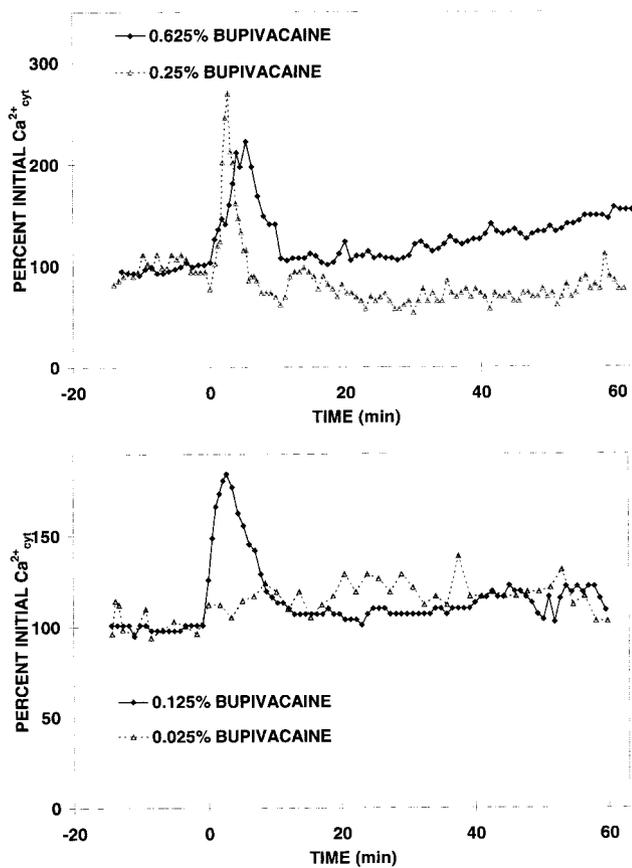


Fig. 2. Effect of bupivacaine on neuronal $\text{Ca}^{2+}_{\text{cyt}}$: Representative single neuron $\text{Ca}^{2+}_{\text{cyt}}$ tracings. Note that 0.625% bupivacaine (19.3 mM) neurons did show moderate late increase in $\text{Ca}^{2+}_{\text{cyt}}$ but much less than equipotent (2.5%) lidocaine. Tris controls are omitted for clarity; all were similar to Tris control illustrated for 5% lidocaine in fig. 1; *i.e.*, flat with minimal increase. Tracings are representative of $N = 5$ to 6 experiments for 0.25 and 0.625% bupivacaine, and of $N = 3$ to 4 experiments for 0.025–0.125% bupivacaine and for all Tris controls; number of neurons per experiment = 17 ± 5 (SD).

exposures of 60 min. Lidocaine greater than 0.5% caused an initial peak in $\text{Ca}^{2+}_{\text{cyt}}$ to approximately twice baseline, which subsided within 10 min. Lidocaine 0.5 and 1.0% returned to near baseline values of $\text{Ca}^{2+}_{\text{cyt}}$ after the initial peak. In contrast, lidocaine 2.5% and 5% caused a sustained increase in $\text{Ca}^{2+}_{\text{cyt}}$ to toxic levels, with some instances of neuronal death by plasma membrane lysis (necrosis) observed within the 60 min experimental period. Tris controls equipotent to the lidocaine and bupivacaine concentrations tested caused minimal changes in $\text{Ca}^{2+}_{\text{cyt}}$ and no neuronal death.

Figure 2 illustrates representative single neuron $\text{Ca}^{2+}_{\text{cyt}}$ responses to equipotent bupivacaine exposures of 60 min, using a potency ratio of 1:4. Bupivacaine 1.25% (equipotent to 5% lidocaine) could not be tested because it is not soluble at pH 7.4 at 37°C. Bupivacaine greater than 0.125% caused an initial $\text{Ca}^{2+}_{\text{cyt}}$ peak similar to that seen with lidocaine, but not a large sustained increase. At the highest bupivacaine concentration that could be tested, 0.625% (19.3 mM), there was generally a

slow increase in $\text{Ca}^{2+}_{\text{cyt}}$ as illustrated in figure 2, with a few neurons increasing $\text{Ca}^{2+}_{\text{cyt}}$ several-fold over 60 min. No neuronal death was observed for any concentration of bupivacaine during 60 min exposure.

Effect of LA on Neuronal $\text{Ca}^{2+}_{\text{cyt}}$

Quantitative Analysis. Qualitative inspection of individual neuronal tracings (figs. 1 and 2) indicated a biphasic response to local anesthetic: A first phase comprising a small increase in $\text{Ca}^{2+}_{\text{cyt}}$ and subsequent decline to near baseline, completed within the first 10 min, and a second phase comprising either a more prolonged, steady increase in $\text{Ca}^{2+}_{\text{cyt}}$ for higher concentrations of lidocaine, or a stable maintenance of near baseline $\text{Ca}^{2+}_{\text{cyt}}$ for the remainder of the 60 min experimental period. Hence, quantitative analysis of average $\text{Ca}^{2+}_{\text{cyt}}$ was performed separately for the time periods of 0–10 min, and 10–60 min.

For 0–10 min, summarized in figure 3, lidocaine 0.1–5% and bupivacaine 0.125–0.625% increased $\text{Ca}^{2+}_{\text{cyt}}$ compared with equipotent Tris controls. Lidocaine 1 and 2.5% caused a greater increase than equipotent bupivacaine. The increase with LA concentration was significant by linear regression for both lidocaine and bupivacaine.

For 10–60 min, summarized in figure 4, the effect of LA was quite different between low and high concentrations. $\text{Ca}^{2+}_{\text{cyt}}$ did not differ from or was less than Tris controls for both lidocaine 1.0% and below and equipotent bupivacaine, and returned to baseline or lower levels. In contrast, lidocaine 2.5 and 5% and bupivacaine 0.625% showed a large increase in $\text{Ca}^{2+}_{\text{cyt}}$ during the 10–60 min period, lidocaine 2.5% more than 0.625% bupivacaine. The increase in $\text{Ca}^{2+}_{\text{cyt}}$ with 5% lidocaine was not greater during 10–60 min than during 0–10 min. However, this may reflect the limits of $\text{Ca}^{2+}_{\text{cyt}}$ monitoring with fura-2. Because the $\text{Ca}^{2+}_{\text{cyt}}$ calibration equation is ratiometric, and the K_d of fura-2 for Ca^{2+} is approximately 220 nM, fura-2 begins to saturate and does not accurately report $\text{Ca}^{2+}_{\text{cyt}}$ values greater than 1,000 nM.¹³

Origin of Increased $\text{Ca}^{2+}_{\text{cyt}}$ Caused by Lidocaine—Effect of Thapsigargin

To test the involvement of the endoplasmic reticulum (ER) in LA-induced $\text{Ca}^{2+}_{\text{cyt}}$ increase, neurons were pretreated prior to LA addition with 100 nM thapsigargin (Tps), which releases and depletes ER Ca^{2+} by inhibiting the ER's ATP-dependent Ca^{2+} transport.²⁸ Representative single neuron tracings are depicted in figure 5, while statistical comparisons of the $\text{Ca}^{2+}_{\text{cyt}}$ averages for 0–10 min and 10–60 min after LA addition are depicted in figure 6. As expected, Tps pretreatment caused a transient increase in $\text{Ca}^{2+}_{\text{cyt}}$ which returned to a plateau level slightly higher than pre-Tps $\text{Ca}^{2+}_{\text{cyt}}$ as $\text{Ca}^{2+}_{\text{cyt}}$ released from the ER was transported out of the cell and sequestered in other organelles. Subsequent addition of

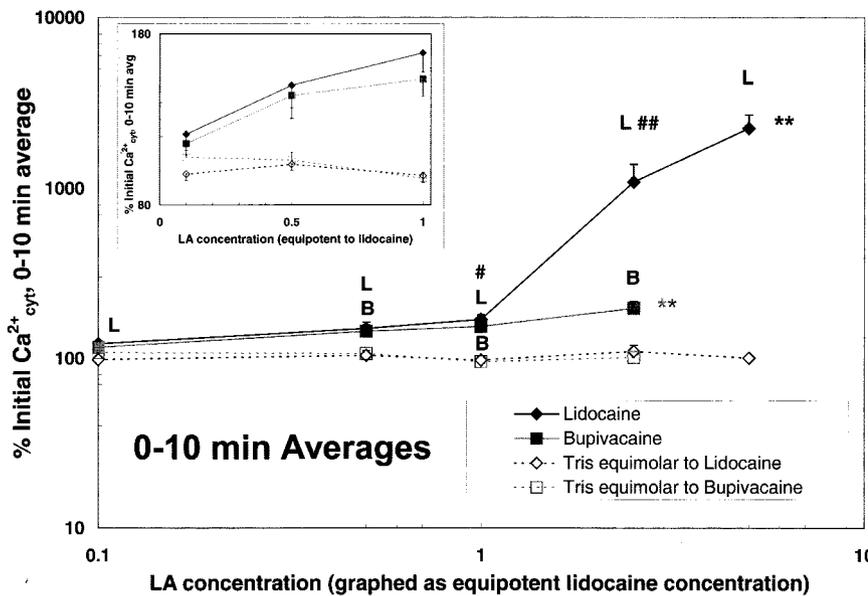


Fig. 3. Effect of local anesthetic (LA) on neuronal Ca^{2+}_{cyt} : Quantitative analysis of average neuronal Ca^{2+}_{cyt} during 0–10 min exposure to LA. Bupivacaine is plotted as its equipotent lidocaine concentration to facilitate comparisons between IAs; e.g., bupivacaine graphed on the x-axis as 1.0% is actually 0.25% bupivacaine. Tris controls are plotted as the LA concentration to which they are equimolar, and for which they serve as a specific control. Note the log-log plot to accommodate the wide range of average Ca^{2+}_{cyt} values. Inset displays non-logarithmic plot of same data for LA equipotent to 0.1–1.0% lidocaine, to illustrate increase of Ca^{2+}_{cyt} with LA concentration during the 0–10 min period. Symbols used to indicate statistical significance: L = $P < 0.001$ lidocaine versus equimolar Tris (same concentration and time); B = $P < 0.001$ bupivacaine versus equimolar Tris (same concentration and time); ## = $P < 0.001$ lidocaine versus bupivacaine (same concentration and time); ** = $P < 0.001$ concentration effect for single LA (linear regression of all concentrations tested).

tion and time); # = $P < 0.05$ lidocaine versus bupivacaine (same concentration and time); ** = $P < 0.001$ concentration effect for single LA (linear regression of all concentrations tested).

LA equipotent to lidocaine 1 or 2.5% caused minimal increase in Ca^{2+}_{cyt} , consistent with the ER being the origin of the initial transient Ca^{2+}_{cyt} peak seen with these LA concentrations in the absence of Tps. In contrast, Tps pretreatment had no effect on the large increase in Ca^{2+}_{cyt} caused by 5% lidocaine for 0–10 min or for 10–60 min, differing from all the other lidocaine and bupivacaine concentrations tested (although drawing quantitative conclusions from such high Ca^{2+}_{cyt} values is limited by the measurement range of fura-2, as described previously [“Effect of LA on Neuronal Ca^{2+}_{cyt} : Quantita-

tive Analysis,” end of third paragraph]). It is likely that 5% lidocaine still elicited an early Ca^{2+} release from the ER, since an early peak was often visible in the individual cell tracings (fig. 1), but that its magnitude was quantitatively insignificant compared to the small portion of the later Ca^{2+}_{cyt} peak which occurred prior to 10 min. Because the initial and later Ca^{2+}_{cyt} peaks frequently overlapped with 5% lidocaine, and the onset time of the later Ca^{2+}_{cyt} peak was variable, a greater differentiation between the two peaks was not feasible. A small minority (4/65) of neurons treated with 2.5% lidocaine had a

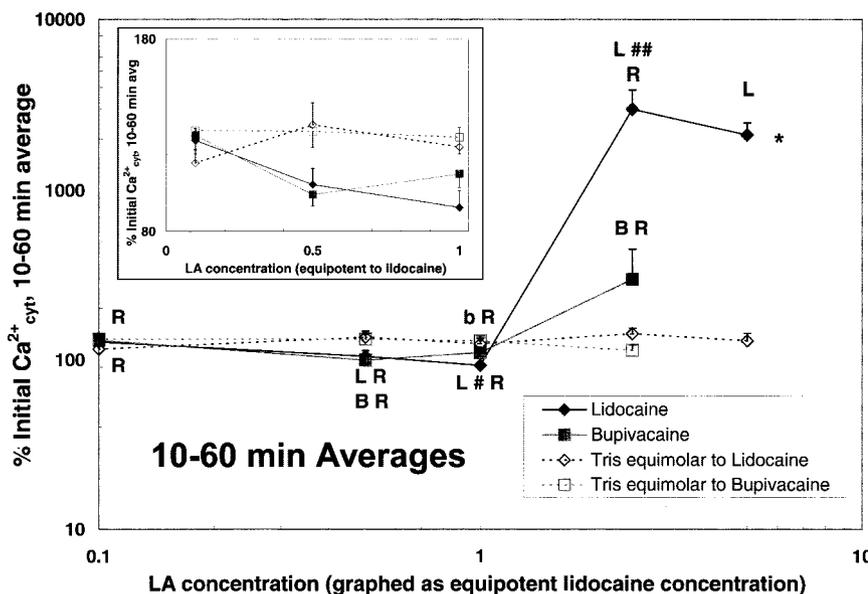


Fig. 4. Effect of local anesthetic (LA) on neuronal Ca^{2+}_{cyt} : Quantitative analysis of average neuronal Ca^{2+}_{cyt} during 10–60 min exposure to LA. Bupivacaine is plotted as its equipotent lidocaine concentration to facilitate comparisons between IAs; e.g., bupivacaine graphed on the x-axis as 1.0% is actually 0.25% bupivacaine. Tris controls are plotted as the LA concentration to which they are equimolar, and for which they serve as a specific control. Note the log-log plot to accommodate the wide range of average Ca^{2+}_{cyt} values. Inset displays non-logarithmic plot of same data for LA equipotent to 0.1–1.0% lidocaine, to illustrate decrease of Ca^{2+}_{cyt} with LA concentration during 10–60 min period (in contrast to increase of Ca^{2+}_{cyt} with LA equipotent to > 1%). Symbols used to indicate statistical significance: L = $P < 0.001$ lidocaine versus equimolar Tris (same concentration and time); b = $P < 0.05$ bupivacaine versus equimolar Tris (same concentration and time); B = $P < 0.001$ bupivacaine versus equimolar Tris (same concentration and time); ## = $P < 0.001$ lidocaine versus bupivacaine (same concentration and time); # = $P < 0.05$ lidocaine versus bupivacaine (same concentration and time); R = $P < 0.001$ 0–10 min versus 10–60 min (same concentration of lidocaine or bupivacaine and same time; shown on 10–60 min graph only for clarity); * = $P < 0.05$ concentration effect for single LA (linear regression of all concentrations tested).

versus equimolar Tris (same concentration and time); B = $P < 0.001$ bupivacaine versus equimolar Tris (same concentration and time); ## = $P < 0.001$ lidocaine versus bupivacaine (same concentration and time); # = $P < 0.05$ lidocaine versus bupivacaine (same concentration and time); R = $P < 0.001$ 0–10 min versus 10–60 min (same concentration of lidocaine or bupivacaine and same time; shown on 10–60 min graph only for clarity); * = $P < 0.05$ concentration effect for single LA (linear regression of all concentrations tested).

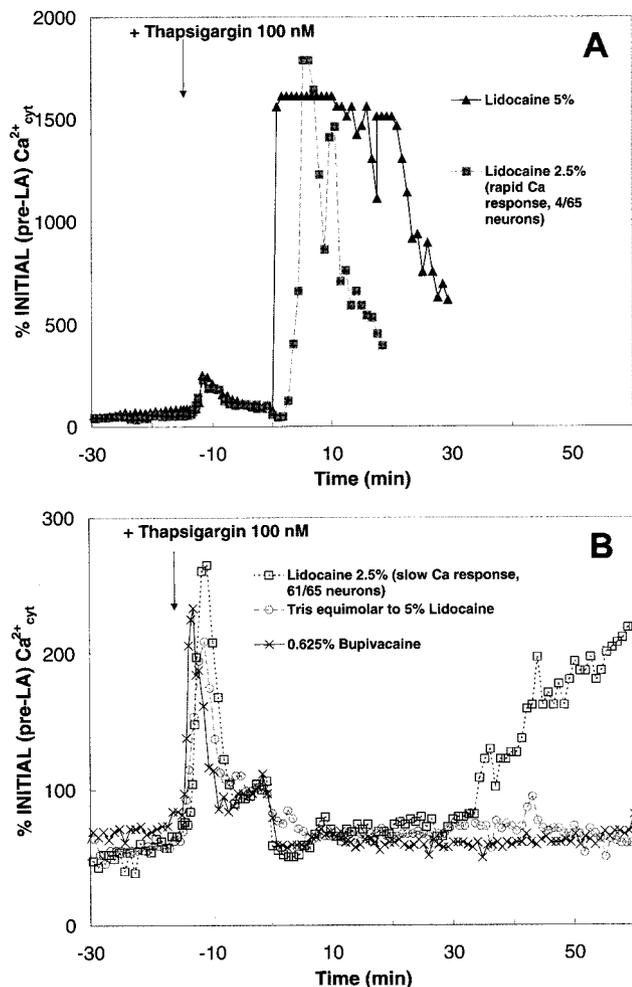


Fig. 5. Effect of thapsigargin pretreatment: representative single neuron Ca^{2+}_{cyt} tracings. LA was added exactly at time 0. Thapsigargin 100 nM pretreatment is indicated approximately by the arrow, with slight variations in time relative to LA addition between experiments. Ca^{2+}_{cyt} values as graphed are normalized to the baseline value in the 5 min preceding LA addition (*i.e.*, after thapsigargin addition). Note: two y-axes for different magnitudes of Ca^{2+}_{cyt} response between (A) and (B), and two types of Ca^{2+}_{cyt} response (rapid, high and slow, low) to 2.5% lidocaine. The rapid, high Ca^{2+}_{cyt} response to 2.5% lidocaine (A) occurred in 4 out of 65 neurons in three experiments. The slow, low Ca^{2+}_{cyt} response to 2.5% lidocaine (B) occurred in the other 61 neurons. The other tracings were selected from 3 to 4 experiments with greater than 50 neurons total for each LA concentration. Neuronal death occurred before 60 min for all neurons treated with 5% lidocaine, and for the neurons treated with 2.5% lidocaine which had a rapid, high calcium response. In the individual neuron tracings presented, the tracing stops at the time of neuronal death. Lidocaine 1% (59 neurons in 3 experiments) and bupivacaine 0.25% (61 neurons in 3 experiments) had the same pattern as shown for 0.625% bupivacaine and Tris control; *i.e.*, little change in Ca^{2+}_{cyt} after LA addition, and are omitted for clarity. LA concentrations equipotent to lidocaine less than 1% were not tested.

Ca^{2+}_{cyt} response similar to that with 5% lidocaine. These may represent rare neurons with preexisting injury or senescence, so that they are less able to deal with an increase of Ca^{2+}_{cyt} . They may also indicate that the dose-response curve for the Tps-independent increase

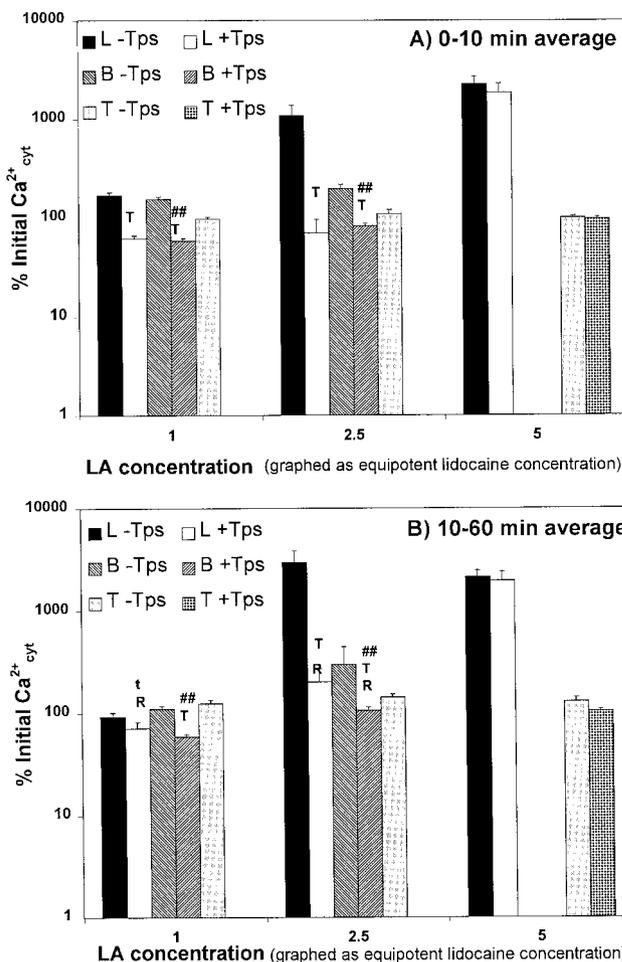


Fig. 6. Effect of thapsigargin 100 nM pretreatment: quantitative analysis of average neuronal Ca^{2+}_{cyt} during (A) 0–10 min, and (B) 10–60 min, exposure to LA. First letter indicates LA treatment: L = lidocaine, B = bupivacaine, T = Tris control equimolar to lidocaine. Modifier indicates thapsigargin pretreatment: –Tps = no thapsigargin pretreatment; +Tps = with thapsigargin pretreatment. Note that these legend symbols are distinct from the symbols used over the bar graphs to indicate statistical significance (listed at end of next paragraph). Data without Tps are the same as in figs. 3 and 4, repeated here to facilitate comparison \pm Tps. Bupivacaine 1.25% equipotent to 5% lidocaine was not tested because of solubility limits at pH 7.4. Since the purpose of these experiments was to determine the effect of Tps on the perturbation of Ca^{2+}_{cyt} by LA, the only Tris control done for +Tps was equimolar to 5% lidocaine (*i.e.*, highest concentration) and is included for illustration only, not for statistical comparisons. Only statistical comparisons specific to Tps are shown on this graph: T = $P < 0.001$ + versus – Tps (same LA, concentration, time period); t = $P < 0.01$ + versus – Tps (same LA, concentration, time period); ## = $P < 0.001$ bupivacaine versus lidocaine (+Tps; same concentration and time period); # = $P < 0.05$ bupivacaine versus lidocaine (+Tps; same concentration and time period); R = $P < 0.001$ 0–10 min versus 10–60 min (+Tps; same LA and concentration); shown only on 10–60 min graph.

in Ca^{2+}_{cyt} with lidocaine is fairly steep at 2.5%, with neurons at 2.5% lidocaine very close to responding as they would to 5% lidocaine, and needing only a small impetus from other random variables to push them to that point. Most neurons treated with 2.5% lidocaine had

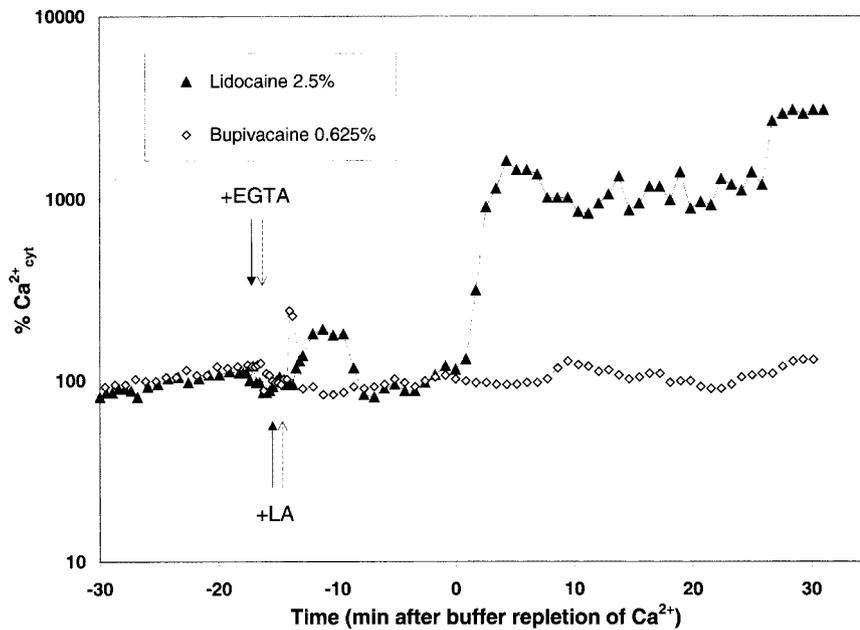


Fig. 7. Effect of local anesthetic (LA) on Ca^{2+} repletion after EGTA-mediated Ca^{2+} depletion: representative single neuron $\text{Ca}^{2+}_{\text{cyt}}$ tracings. Calcium values on y-axis are normalized to the average $\text{Ca}^{2+}_{\text{cyt}}$ for 5 min prior to buffer Ca^{2+} repletion. Initial buffer was 2 mM Ca^{2+} . Downward arrows indicate addition of EGTA to yield 4 mM EGTA, so that available extracellular Ca^{2+} was approximately 0. Upward arrows indicate addition of LA. Solid arrows indicate lidocaine, dotted arrows indicate bupivacaine experiment. At 0 min, Ca^{2+} was added to yield 4 mM, or approximately 2 mM unchelated extracellular Ca^{2+} . Note log scale on y-axis. In all experiments, EGTA was added 15 to 16 min prior to Ca^{2+} repletion, and LA was added 14 to 15 min prior to Ca^{2+} repletion. In control experiments, there was no increase in $\text{Ca}^{2+}_{\text{cyt}}$ after the initial small peak when Ca^{2+} was not repleted and the neurons were incubated in Ca^{2+} -free buffer for 45 min after addition of LA (data not shown).

an intermediate response: a slow, delayed increase in $\text{Ca}^{2+}_{\text{cyt}}$ of lesser magnitude, doubling or tripling by 60 min, and were viable at 60 min. This suggests that higher concentrations of lidocaine (2.5 and 5%), unlike bupivacaine and the lower concentration of lidocaine tested (1%), increase $\text{Ca}^{2+}_{\text{cyt}}$ from a source other than the ER.

Origin of Increased $\text{Ca}^{2+}_{\text{cyt}}$ Caused by Lidocaine—Effect of Extracellular Calcium

To test the effect of higher dose LA on Ca^{2+} influx from extracellular fluid, extracellular buffer was depleted of Ca^{2+} , neurons exposed to LA, and extracellular buffer then replenished with Ca^{2+} . Representative single neuron tracings are shown in figure 7, and quantitative summaries of all experiments in figure 8. In the absence of extracellular Ca^{2+} , both lidocaine 2.5% and bupiva-

caine 0.625% caused a small increase in $\text{Ca}^{2+}_{\text{cyt}}$, indicating that both LAs release Ca^{2+} from intracellular stores, consistent with the Tps experiments described previously. When Ca^{2+} was added back to the extracellular buffer (at time 0 in the figure), there was no effect in neurons exposed to bupivacaine. For neurons exposed to lidocaine 2.5% and 5%, however, there was an immediate and sustained ten-fold increase in $\text{Ca}^{2+}_{\text{cyt}}$, suggesting that lidocaine greater than 2.5% causes a large increase in plasma membrane permeability to Ca^{2+} .

Neuronal Death Caused by Local Anesthetic

The experiments described here are not optimized to study neuronal death, since they follow relatively few numbers of neurons, but to determine quantitative changes in $\text{Ca}^{2+}_{\text{cyt}}$ over time in each neuron. Neverthe-

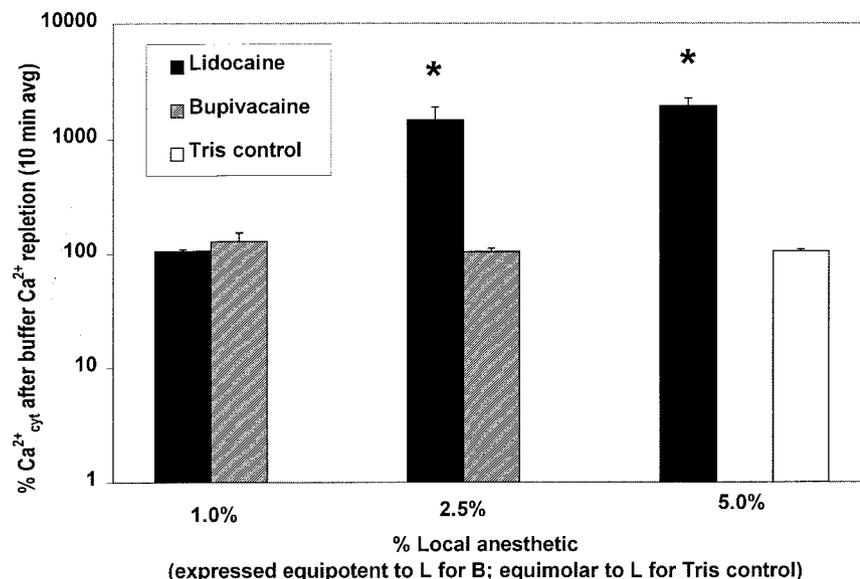
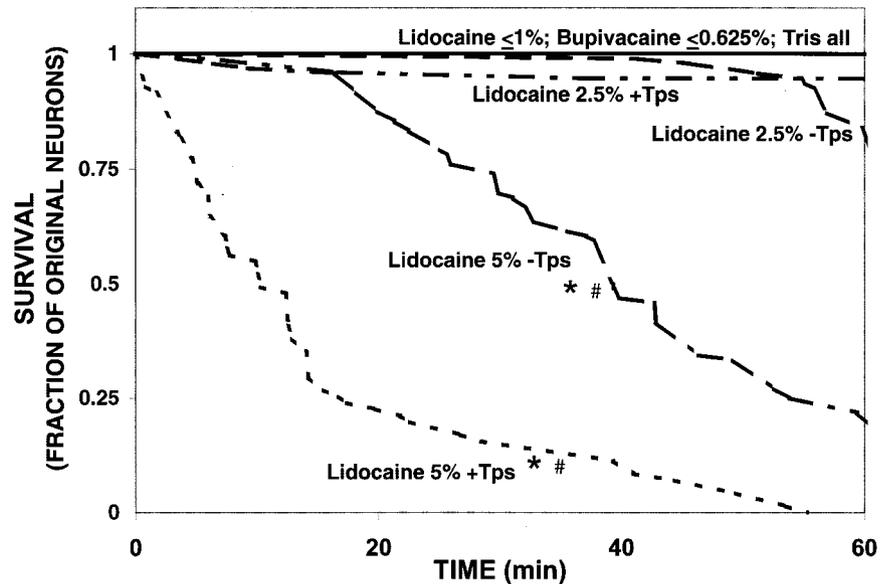


Fig. 8. Effect of local anesthetic (LA) on Ca^{2+} repletion after EGTA-mediated Ca^{2+} depletion: Quantitative analysis. * $P < 0.0001$ versus 1% lidocaine, all bupivacaine, and Tris control. $P > 0.05$ for all other comparisons. $N = 3$ experiments for bupivacaine and lidocaine greater than 1%; $N = 2$ experiments for lidocaine 1% and Tris control; N greater than 15 neurons for each experiment. Y-axis is $\text{Ca}^{2+}_{\text{cyt}}$ averaged over the 10 min following Ca^{2+} repletion (0–10 min on x-axis in fig. 7), normalized to the $\text{Ca}^{2+}_{\text{cyt}}$ averaged over the 5 min preceding Ca^{2+} repletion. Averages and normalization were done separately for each neuron, then experimental averages calculated with equal weighting for each experiment and comparisons made by analysis of variance with Tukey correction for *post hoc* comparisons.

Fig. 9. Cumulative survival curves for 60 min for neurons in all LA experiments \pm pretreatment with 100 nM thapsigargin. (Dotted line) Lidocaine 5% +thapsigargin; N = 71 neurons; (Dash-dot line) lidocaine 5% -thapsigargin; N = 96 neurons; (dash-dot-dot line) lidocaine 2.5% +thapsigargin; N = 75 neurons; (Dashed line) lidocaine 2.5% -thapsigargin; N = 94 neurons. (Solid line) lidocaine less than 1%; Bupivacaine less than 0.625%, Tris all concentrations; N greater than 58 neurons for each LA and Tris concentration; no neuronal death observed within 60 min. * $P < 0.0001$ for - versus + thapsigargin pretreatment, 5% lidocaine; # $P < 0.0001$ for 5% lidocaine versus less than 5% lidocaine, all bupivacaine, and all Tris controls (all \pm thapsigargin)



less, it is essential to determine the time of death in each neuron in these experiments, because measurement of $\text{Ca}^{2+}_{\text{cyt}}$ is meaningful only when neurons have not undergone necrotic death and still have an intact plasma membrane.¹³ Survival curves are plotted in figure 9. Lidocaine 5% by itself caused significant neuronal necrosis within the 60 min protocol period, with a mean survival time of 42 min. Pretreatment with Tps increased neuronal death significantly, with a mean survival time of 15 min. Although neuronal death was observed with 2.5% lidocaine \pm Tps, the effect of Tps was not statistically significant at 2.5% lidocaine, and the incidence of neuronal death with 2.5% lidocaine did not differ statistically from Tris controls. No death was observed within 60 min in any experiment with lidocaine less than 1%, bupivacaine less than 0.625%, or Tris control.

Discussion

Our data establish that the highest concentration of lidocaine clinically available (5%) can cause necrotic cell death within 60 min in a neuronal cell culture model. Our data also show that clinical concentrations of lidocaine seen with well-mixed subarachnoid administration, less than 0.5%,²⁹ do not cause necrotic neuronal death within 60 min nor major alterations in calcium homeostasis, consistent with the clinical experience that the vast majority of lidocaine spinal anesthetics do not cause lasting neural injury. Translation of any cell culture model to the clinical situation is always challenging, because the model does not mimic perfectly the clinical situation. One significant difference of our model from clinical practice is that we maintained a constant concentration of LA during the 60 min exposure period, whereas *in vivo* the CSF concentration would decrease

with time as the LA mixed and diffused out of the CSF. We chose this method to maximize reproducibility with a constant LA concentration, and to model the clinical situation of poor LA mixing that appears to increase risk of neurotoxicity. However, our model may overstate the toxicity that would be seen with a given LA concentration that decreased over time.

A new finding reported here is that both lidocaine greater than 0.5% and bupivacaine greater than 0.125% cause an initial, short-lived (approximately 5 min), moderate increase in $\text{Ca}^{2+}_{\text{cyt}}$. At lower LA concentrations, $\text{Ca}^{2+}_{\text{cyt}}$ then returns to normal levels, then decreases below control levels, and death is not observed within our 60 min experimental period. Whether this $\text{Ca}^{2+}_{\text{cyt}}$ increase or the subsequent decrease causes any clinically significant effect is unknown. However, there are also known examples of major physiologic changes being triggered by a similar, single, short-lived increase in $\text{Ca}^{2+}_{\text{cyt}}$; e.g., the metaphase-anaphase transition.³⁰ Short periods of increased $\text{Ca}^{2+}_{\text{cyt}}$ similar to those seen in our experiments (several minutes) can also be associated with synaptic changes affecting neuronal memory and excitability.³¹ A speculation which is consistent with the observed occurrence of the clinical syndrome of Transient Neurologic Symptoms (TNS) with low concentrations of lidocaine, ($\leq 0.5\%$)³² and the modulating effects of spinal $\text{Ca}^{2+}_{\text{cyt}}$ on pain processing,³³ is that the initial short-lived increase in $\text{Ca}^{2+}_{\text{cyt}}$ may initiate a period of increased electrical responsiveness by the neuron, causing hyperalgesia. However, we found no differences between bupivacaine and lidocaine in their effects on $\text{Ca}^{2+}_{\text{cyt}}$ at concentrations equipotent to 0.1% and 0.5% lidocaine, and only a small although statistically significant difference at 1% lidocaine, while there is a dramatic difference in clinical incidence of TNS between lidocaine and bupivacaine.³⁴

The source of the initial short-lived, modest increase in $\text{Ca}^{2+}_{\text{cyt}}$ with lidocaine greater than 0.5% and bupivacaine greater than 0.125% appears to be the ER. The ER is a major regulator of $\text{Ca}^{2+}_{\text{cyt}}$, releasing Ca^{2+} to the cytoplasm or sequestering it in response to multiple stimuli transduced through its IP_3 and ryanodine receptors. Our data are consistent with a large body of work in muscle showing release of Ca^{2+} from the sarcoplasmic reticulum by LAs, at last partially modulated by the ryanodine receptor, although tissue differences may limit the applicability to neurons.^{35,36} The subsequent large, sustained increase in $\text{Ca}^{2+}_{\text{cyt}}$ seen with some neurons exposed to 2.5% lidocaine, and all neurons exposed to 5% lidocaine, does not originate in the ER, but represents influx of $\text{Ca}^{2+}_{\text{cyt}}$ from the outside of the neuron through the plasma membrane. Our data do not address whether this is an effect on existing ion channels, or a direct effect on the plasma membrane lipid bilayer, although both are plausible given the known interaction of LAs with multiple ion channels and the amphipathic character of LAs which may affect lipid bilayer permeability.³⁷

It may be of some concern, and requires further study, that both lidocaine and bupivacaine, at low as well as high concentrations, caused an initial release of Ca^{2+} from the ER. Recent data have suggested that depletion of ER Ca^{2+} stores is by itself a severe form of cellular stress, irrespective of $\text{Ca}^{2+}_{\text{cyt}}$ levels.³⁸ Although with our protocol we did not observe necrosis in neurons exposed to lower concentrations of local anesthetic, we cannot exclude the possibility that delayed neuronal death occurred later than 60 min at lower concentrations.

While our data do not yet establish a change in $\text{Ca}^{2+}_{\text{cyt}}$ homeostasis as the initial, proximate cause of LA neurotoxicity, our data do establish an important role for the later, sustained increase in $\text{Ca}^{2+}_{\text{cyt}}$ in the manifestation of LA neurotoxicity: $\text{Ca}^{2+}_{\text{cyt}}$ homeostasis is altered by LAs, the magnitude of alteration parallels the incidence of neuronal death, the incidence of neuronal death increases when the ER is rendered unable to sequester Ca^{2+} prior to lidocaine treatment, and lidocaine and bupivacaine differ in their effects on $\text{Ca}^{2+}_{\text{cyt}}$. Sustained, high magnitude increases in $\text{Ca}^{2+}_{\text{cyt}}$ as seen with 2.5 and 5% lidocaine are generally associated with toxicity.^{12,39} It is probable that a longer exposure or observation time on larger numbers of cells would reveal significant neuronal death with 2.5% lidocaine as well as with 5%. This can better be determined with a different experimental approach (e.g., flow cytometry) than the one chosen here (digitized video fluorescence microscopy), which allows continuous monitoring of a small number of cells.

There was a clear, five- to ten-fold difference between equipotent 2.5% lidocaine and 0.625% bupivacaine in terms of the later, sustained $\text{Ca}^{2+}_{\text{cyt}}$ response elicited by the LA, consistent with the greater clinical frequency of serious neural injury after lidocaine than bupivacaine.² There was detectable neuronal death with 2.5% lido-

caine and not with equipotent 0.625% bupivacaine in our experiments, although the different survival curves were not statistically different during our 60 min protocol. Our inability to compare 5% lidocaine with equipotent bupivacaine illustrates another factor that may be responsible for part or all of the apparent lesser toxicity of bupivacaine clinically: its decreased solubility compared with lidocaine, such that a bupivacaine preparation equipotent to 5% lidocaine is not available and would not be soluble at physiologic pH. Even commercially available 0.75% bupivacaine must be acidified to pH approximately 4 to stay in solution; we cannot consistently prepare solutions of 0.75% bupivacaine without precipitates forming at pH 7.4 at 37°C.

The effect of Tps on neuronal death suggests hypotheses about both the early and late LA-induced $\text{Ca}^{2+}_{\text{cyt}}$ peaks. Tps pretreatment eliminated the initial, small, transient $\text{Ca}^{2+}_{\text{cyt}}$ peak but did not decrease toxicity, suggesting that the early peak is not associated with acute toxicity within 60 min. This is consistent with our finding that the initial $\text{Ca}^{2+}_{\text{cyt}}$ peak was seen with non-toxic concentrations of both lidocaine and bupivacaine. Tps pretreatment increased neuronal death with 5% lidocaine only, and 5% lidocaine was the only LA concentration tested where Tps pretreatment did not decrease $\text{Ca}^{2+}_{\text{cyt}}$ (fig. 6), suggesting that the greater than ten-fold late, sustained elevation in $\text{Ca}^{2+}_{\text{cyt}}$ seen with 5% lidocaine is mechanistically involved in its neurotoxicity. Tps is quite specific as a tool to deplete ER Ca^{2+} and determine whether a $\text{Ca}^{2+}_{\text{cyt}}$ peak originates from the ER, as the early peak with LA does. However, the effect of Tps is not well localized as a cause of cytotoxicity. Tps has several effects on intracellular Ca^{2+} homeostasis which by themselves, independent of $\text{Ca}^{2+}_{\text{cyt}}$, can increase toxicity: (1) Tps depletes the ER of Ca^{2+} ^{28,38}; (2) By effectively preventing ER uptake of $\text{Ca}^{2+}_{\text{cyt}}$, Tps can increase the demand on other intracellular $\text{Ca}^{2+}_{\text{cyt}}$ handling systems,⁴⁰ particularly (A) mitochondria, with consequent elevation in mitochondrial Ca^{2+} and demands on mitochondrial energy stores, and (B) plasma membrane ion pumps, with increased demand for ATP for export of $\text{Ca}^{2+}_{\text{cyt}}$.

The late decrease in $\text{Ca}^{2+}_{\text{cyt}}$ after the initial increase, seen with 0.5% and 1% lidocaine and equipotent bupivacaine, is consistent with the known Na^+ and Ca^{2+} channel blocking properties of lidocaine, and with the protective effect of Na^+ blockade seen in some models of ischemic neuronal injury.^{41,42} However, a similar decrease was not seen with 0.1% lidocaine, which is still far above the ED_{50} for Na^+ channel block, suggesting that factors other than Na^+ and Ca^{2+} channel blockade are responsible.

Our data are generally consistent with those of Gold *et al.*,¹⁹ who used primary cultures of adult rat dorsal root ganglion neurons to assess the neurotoxicity of lidocaine. Their electrophysiologic studies showed that lido-

caine greater than approximately 0.25% irreversibly depolarized neurons, consistent with a neurotoxic effect not mediated by Na^+ channel blockade. They also tested the effect of 30 s pulses of lidocaine on $\text{Ca}^{2+}_{\text{cyt}}$ and the toxicity of a 15 min exposure to lidocaine followed by a 60 min recovery without lidocaine. Lidocaine pulses caused an increase in $\text{Ca}^{2+}_{\text{cyt}}$ with an ED_{50} of 21 mM (approximately 0.5%) lidocaine. Prolonged monitoring of $\text{Ca}^{2+}_{\text{cyt}}$ beyond the 30 s pulse was not done, nor was the effect of bupivacaine assessed. The amplitude of the $\text{Ca}^{2+}_{\text{cyt}}$ response to the lidocaine pulse was diminished, but not eliminated, with nominally Ca^{2+} -free buffer, implicating both intracellular and extracellular Ca^{2+} as sources of the lidocaine-induced increase in $\text{Ca}^{2+}_{\text{cyt}}$.

Gold *et al.*¹⁹ reported that both 30 mM (0.8%) and 100 mM (2.7%) lidocaine were more toxic than control after 15 min exposure and 60 min recovery, giving 22% and 32% neuronal death, respectively, which is a greater toxicity than we observed. There are two likely reasons for this discrepancy. First is the difference between neuronal cells used. Their primary cultures of acutely isolated DRG neurons were more recently traumatized by dissection and isolation than our continuous cell line. Roughly 7% of the neurons exposed to control by Gold *et al.*¹⁹ died, *versus* none of the neurons in our control experiments. Recent studies have documented that axotomy, an inevitable consequence of DRG excision and dissociation, rapidly alters key properties of these neurons.⁴³ Also, continuous cell lines such as we used tend to be more resistant to many types of injury because they are neoplastic, although this introduces another problem in extrapolating to *in vivo* neurons.⁴⁴ The differences between our toxicity data and that of Gold *et al.*¹⁹ are consistent with the differences between the neuronal cells used. Although extrapolation from cell culture to *in vivo* is always difficult, it is reasonable to suggest that the true susceptibility of spinal neurons to lidocaine toxicity *in vivo* may lie between that of our data and that of Gold *et al.*¹⁹

Second, Gold *et al.*¹⁹ reported that significant numbers of neurons exposed to lidocaine lifted from the coverslips they were cultured on and were lost to analysis, making quantitation of cell death problematic. While they partially compensated for this by analyzing the number of dead neurons as a percent of remaining adherent neurons, it is unlikely that the population of neurons that lifted had the same characteristics as the neurons that remained adherent. We also assayed individual neurons under the microscope, but used glass coverslips coated with a substrate of high-density poly-D-lysine, which we have found to maintain adhesion of essentially all neurons for at least 60 min during exposure to lidocaine. We were unable to attain cultures stably adhesive during LA exposure using collagen or poly-DL-ornithine with or without laminin or other protein supplementation. This is in itself most likely an

indication of LA toxicity, as rounding of cells and detachment from substratum attachment are typical, nonspecific indicators of acute cellular injury.^{45,46}

In conclusion, lidocaine is clearly neurotoxic and elevates $\text{Ca}^{2+}_{\text{cyt}}$ to toxic levels at clinically available concentrations which might be achieved with poor CSF mixing, in a neuronal cell culture model which eliminates vascular and other systemic effects. Lower concentrations of lidocaine and all concentrations of bupivacaine alter $\text{Ca}^{2+}_{\text{cyt}}$ homeostasis for several minutes, but without an immediate neurotoxic effect within 60 min. Both LAs initially release Ca^{2+} from the ER, but only lidocaine 2.5% or 5% also causes a sustained influx of Ca^{2+} through the plasma membrane.

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