

Lidocaine Toxicity in Primary Afferent Neurons from the Rat¹

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ABSTRACT

Evidence from both clinical studies and animal models suggests that the local anesthetic, lidocaine, is neurotoxic. However, the mechanism of lidocaine-induced toxicity is unknown. To test the hypothesis that toxicity results from a direct action of lidocaine on sensory neurons we performed *in vitro* histological, electrophysiological and fluorometrical experiments on isolated dorsal root ganglion (DRG) neurons from the adult rat. We observed lidocaine-induced neuronal death after a 4-min exposure of DRG neurons to lidocaine concentrations as low as 30 mM. Consistent with an excitotoxic mechanism of neurotoxicity, lidocaine depolarized DRG neurons at concentrations that induced cell death ($EC_{50} = 14$ mM). This depolarization occurred even though voltage-gated sodium currents and action potentials were blocked effectively at much lower concentrations. (EC_{50} values for lidocaine-induced block of tetrodo-

toxin-sensitive and -resistant voltage-gated sodium currents were 41 and 101 μ M, respectively.) At concentrations similar to those that induced neurotoxicity and depolarization, lidocaine also induced an increase in the concentration of intracellular Ca^{++} ions ($[Ca^{++}]_i$; $EC_{50} = 21$ mM) via Ca^{++} influx through the plasma membrane as well as release of Ca^{++} from intracellular stores. Finally, lidocaine-induced neurotoxicity was attenuated significantly when lidocaine was applied in the presence of nominally Ca^{++} -free bath solution to DRG neurons preloaded with 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA). Our results indicate: 1) that lidocaine is neurotoxic to sensory neurons; 2) that toxicity results from a direct action on sensory neurons; and 3) that a lidocaine-induced increase in intracellular Ca^{++} is a mechanism of lidocaine-induced neuronal toxicity.

Recent reports of neurologic injury after continuous spinal anesthesia suggest that clinically relevant concentrations of local anesthetics such as lidocaine can cause nerve injury (Auroy *et al.*, 1997; Rigler *et al.*, 1991). Specifically, there is evidence that Cauda Equina syndrome, characterized by incontinence, perineal sensory loss and motor weakness in the legs, is the result of neurotoxicity associated with the spinal administration of local anesthetics (Rigler *et al.*, 1991).

Studies of local anesthetic-induced nerve injury have eliminated several possible mechanisms of toxicity. For example, glucose, included in the local anesthetic solution to control the spread of spinally administered agents, is not neurotoxic (Sakura *et al.*, 1995b), which suggests that toxicity results from an action of the local anesthetic itself. In addition, Lambert and colleagues (1994) demonstrated that irreversible conduction block induced by lidocaine in an isolated frog nerve is not the result of either a failure to wash the local anesthetic from the nerve, or a breakdown of the nerve membrane integrity. Finally, Sakura and colleagues (1995a) concluded that local anesthetic-induced neurotoxicity *in vivo*

does not result from a blockade of voltage-gated sodium channels.

The aim of the present study was to determine whether lidocaine has direct neurotoxic effects on sensory neurons and to investigate the possible involvement of Ca^{++} in this effect. To address this aim, we performed *in vitro* histological, electrophysiological and ratiometric fluorometrical experiments on isolated sensory neurons from the adult rat. We observed lidocaine-induced neuronal death after exposure of cultured DRG neurons to lidocaine concentrations as low as 30 mM for as little as 4 min. In addition, we provide evidence which indicates that lidocaine-induced increase in the intracellular concentration of free Ca^{++} ion ($[Ca^{++}]_i$) contributes to lidocaine-induced neuronal death.

Methods

Cell culture. Primary cultures of dissociated adult rat DRG neurons were prepared by methods described previously (Gold *et al.*, 1996a). Male Sprague-Dawley rats (150–250 g, Bantin and Kingman, Fremont CA) were anesthetized deeply with an i.p. injection of Na-pentobarbital (60 mg/kg); lumbar (L_1 – L_6). DRG were removed, and rats subsequently were sacrificed by an overdose of Na-pentobarbital. DRGs were desheathed in ice-cold MEM-BS composed of

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ABBREVIATIONS: BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; DMSO, dimethyl sulfoxide; DRG, dorsal root ganglion; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; PBS, phosphate-buffered saline; TTX-R I_{Na} , tetrodotoxin-resistant sodium current; TTX-S I_{Na} , tetrodotoxin-sensitive sodium current.

90% minimal essential medium (MEM; Gibco BRL, Gaithersburg, MD), 10% heat-inactivated fetal bovine serum and 1000 U/ml each of penicillin and streptomycin. DRGs were then incubated 120 min at 37°C in MEM-BS, to which collagenase P (Boehringer Mannheim, Indianapolis, IN) was added to a final concentration of 0.125%. DRGs then were incubated 10 min at 37°C in Ca⁺⁺- and Mg⁺⁺-free Hanks' balanced salt solution containing (in g/l): glucose, 1; KCl, 0.4; NaCl, 8.0; KH₂PO₄, 0.06; Na₂HPO₄·7H₂O, 0.09; Phenol Red, 0.01; NaHCO₃, 0.35, and 0.25% trypsin (Worthington, Bristol, UK) and 0.025% ethylenediaminetetraacetic acid (Sigma, St Louis, MO). Then trypsin activity was inhibited by the addition of MEM-BS containing 0.125% MgSO₄ and 80 µg/ml soybean trypsin inhibitor (Sigma), and DRGs were dissociated by trituration with a fire-polished Pasteur pipette. DRG cells were plated onto glass coverslips, previously coated by a solution of 5 µg/ml mouse laminin (Gibco BRL) and 0.1 mg/ml poly-DL-ornithine (Sigma). The cells were incubated in MEM-BS at 37°C, 3% CO₂ and 90% humidity, and were studied between 6 and 24 hr after plating.

Cell survival. Trypan blue exclusion was used to determine the effects of lidocaine on DRG neuron survival. After 15 to 20 hr incubation, coverslips were placed in chambers (500 µl volume) continuously perfused with normal bath solution (table 1, BS 1) or nominally Ca⁺⁺-free bath solution (table 1, BS 3). The perfusion solution was changed to a solution containing lidocaine (1–100 mM) or choline (30–100 mM) (table 1, BS 1 or BS 3 containing lidocaine or choline) 3 to 5 min later. After exposure to test solutions, the perfusion solution was changed again to normal bath solution and the coverslips were perfused continuously for 1 hr. Coverslips were then placed in trypan blue solution (0.1–0.4%) for 10 min, briefly rinsed in normal bath solution and then placed in chilled 4% paraformaldehyde (in PBS) for 30 min at 4°C. Finally, coverslips were rinsed in PBS, dehydrated and mounted on glass slides. All solutions used before fixation were at room temperature.

To determine whether 30 mM and 100 mM choline solutions were adequate controls for the possible effects associated with the osmolality of the 30 mM and 100 mM lidocaine solutions, the osmolality of all solutions was measured with a vapor pressure osmometer (Wescor, Logan, UT; model 5520). The osmolality of the 30 mM lidocaine and choline solutions were both 325 ± 4 mOsm, whereas that of the 100 mM lidocaine and choline solutions were both 444 ± 6 mOsm.

One set of cell survival experiments was performed on cells preloaded with BAPTA. To load cells with BAPTA, coverslips were soaked for 30 min in normal bath solution containing 1 mM BAPTA-AM (Molecular Probes, Eugene, OR) and 0.025% pluronic acid (Molecular Probes). BAPTA-AM was diluted from a 100 mM stock solution prepared with DMSO. After exposure to BAPTA-AM-containing bath solution, coverslips were placed in normal bath solution for 20 min before testing.

TABLE 1
Electrode and bath solutions

Salt	ES ^a		BS ^b		
	1	2	1	2	3
	mM		mM		
NaCl	–	10	130	35	130
KCl	140	–	3	–	3
CsCl	–	140	–	–	–
CaCl ₂	1	1	2.5	0.1	–
MgCl ₂	2	2	0.6	5	0.6
HEPES	10	10	10	10	10
Glucose	–	–	10	10	10
Choline-Cl	–	–	–	65	–
TEA-Cl ^c	–	–	–	30	–
EGTA ^d	11	11	–	–	–

^a Electrode solution.

^b Bath solution.

^c Tetraethylammonium-chloride.

^d Ethyleneglycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid.

Cell survival was quantified by counting the number of cells stained with trypan blue relative to the total number of cells along a single pass through a diameter of the coverslip at ×10 magnification. Cell phenotype was determined based on morphology; DRG neurons, which have a relatively large (>10 µm diameter) and distinctly spherical cell body, are distinguished easily from non-neuronal cells. Cells were visualized by Nomarsky optics. The individual who scored the slides was blinded to the experimental manipulation.

A 15-min exposure to test agents was used in most experiments to facilitate comparison of our data with the data obtained with frog sciatic nerve (Lambert *et al.*, 1994). A 1-hr wash period between lidocaine exposure and trypan blue staining was used to ensure that trypan blue staining reflected an irreversible decrease in cell viability rather than a transient decrease in plasma membrane integrity.

Electrophysiology. To study effects of lidocaine on the electrophysiological properties of DRG neurons, conventional whole-cell voltage-clamp techniques were used. Patch pipettes filled with electrode solution (ES, table 1) had resistance of 1 to 4 megohm. The recording chamber (500-µl volume) was perfused continuously (1–2 ml/min) with bath solution. All experiments were performed at room temperature (21–24°C).

For current-clamp experiments an electrode solution with 140 mM KCl was used (table 1, ES 1) in the presence of normal bath solution (table 1, BS 1). To record voltage-gated Na⁺ currents in isolation, KCl in the electrode solution was replaced by CsCl (table 1, ES 2). To maintain control of the membrane potential while recording voltage-gated Na⁺ currents, the concentration of Na⁺ in the bath solution was reduced (table 1, BS 2). All components of the bath and electrode solutions were obtained from Sigma (St. Louis, MO).

Cells were voltage- or current-clamped by use of an Axopatch 1B amplifier (Axon Instruments, Foster City, CA). Data were filtered with a 4-pole Bessel filter (see figure legends) and digitized. Series resistance was estimated from the settling rate of the voltage clamp and the membrane capacitance. Estimates for series resistance ranged between 0.3 and 5 megohm. Series resistance was compensated (>80%) by use of amplifier circuitry. Data obtained from neurons in which uncompensated series resistance resulted in voltage-clamp errors greater than 5 mV were discarded from further analysis. A P/4 protocol was used for leak subtraction; to ensure the linearity of leakage currents around the holding potential for the P/4 protocol, the resting conductance for each cell was determined at potentials ranging from –100 to –60 mV. Data were fit by a nonlinear least-square method.

Dose-response data were fit by a Michaelis-Menten equation that related fractional inhibition of current, F , to drug concentration: $F = [1 - (I_{\text{drug}}/I_{\text{control}})] = [(F_{\text{max}} \times D^n)/(D^n + EC_{50}^n)]$, characterized by EC₅₀ (concentration of drug producing a half-maximal effect), F_{max} (maximum fraction of inhibition) and n , a Hill coefficient used to account for possible cooperativity. I_{drug} = the peak current in the presence of lidocaine; I_{control} = the peak current in the absence of drug; and D = the lidocaine concentration. A similar equation was fit to the dose-response relationship of lidocaine-induced membrane depolarization. To compare the effects of different concentrations of lidocaine on the resting membrane potential of DRG neurons, we calculated the fractional change in membrane potential induced by each concentration of lidocaine in each neuron. The fractional change in membrane potential was determined by dividing the difference between the membrane potential at the start of the experiment (V_{rest}) and the membrane potential in the presence of a given concentration of lidocaine (V_{ldo}) by the difference between V_{rest} and the membrane potential in the presence of 100 mM lidocaine ($V_{100 \text{ lido}}$): fractional depolarization (F_d) at each concentration of lidocaine = $(V_{\text{rest}} - V_{\text{ldo}})/(V_{\text{rest}} - V_{100 \text{ lido}})$. The equation used to fit the dose dependence of depolarization was $F_d = D^n/(D^n + EC_{50}^n)$.

Ratiometric fluorimetry. After 15 to 20 hr in culture, cells were loaded 10 min with 2.5 µM Fura-2 (with 0.025% pluronic acid, Molecular Probes), after which cells were placed in normal bath solution (table 1) for 10 min and then placed in a chamber continu-

ously perfused at 2 ml/min with normal bath solution (table 1, BS 1). Fura-2 fluorescence was measured with an ICCD camera (Solamere Technology, Salt Lake City UT). Data were analyzed and presented as a Fura-2 fluorescence ratio, because this ratio is directly proportional to $[Ca^{++}]_i$ for concentrations of Ca^{++} less than 10% of the saturating concentration for Fura-2 ($\sim 39 \mu M$). We were able to readily detect increases in $[Ca^{++}]_i$, as demonstrated by the increase in the Fura-2 fluorescence ratio in response to the bath application of lidocaine and 50 mM K^+ . In addition, we were able to readily detect decreases in $[Ca^{++}]_i$, as demonstrated by a decrease in the fluorescence ratio in response to the application of nominally Ca^{++} -free bath solution (table 1, BS 3). Therefore, the resting $[Ca^{++}]_i$ within the DRG neurons we have studied is neither close to the saturating concentration for Fura-2, nor below the threshold for which increase in $[Ca^{++}]_i$ may be detected (~ 5 nM in our system).

Statistics. Data are presented as a mean \pm standard error of the mean (S.E.M.). A one-way analysis of variance was used to determine the presence of statistically significant differences between groups. When a significant group effect was detected, *post hoc* comparisons were performed with a Scheffe test.

Results

Cell death. A 15-min exposure to lidocaine induced death of cultured DRG neurons as assayed by failure to exclude trypan blue (see fig. 1). This effect was dose-dependent, increasing dramatically at lidocaine concentrations greater than 10 mM. The number of neurons stained with trypan blue 1 hr after a 15-min exposure to 30 mM lidocaine was larger than the number of neurons stained after a 15-min exposure to 100 mM lidocaine (fig. 1A). In contrast to the dose-dependent increase in the number of neurons killed after a 15-min exposure to lidocaine, when data are analyzed for neurons killed as a percentage of the total neurons counted (fig. 1B), the percentage of neurons killed after exposure to 100 mM lidocaine was larger than that observed after exposure to 30 mM lidocaine. The difference between the dose dependence of the number of neurons killed *versus* percentage of neurons killed reflects the fact that there was a dose-dependent decrease in the number of neurons remaining on the coverslip.

The number of neurons that died in the presence of 30 mM lidocaine and the percentage of neurons that died in the presence of 100 mM lidocaine were significantly larger than the number or percentage of neurons that died in the presence of either 1 mM lidocaine or the iso-osmotic choline control (fig. 1, A and B, $P < .01$). The number of neurons that died in the presence of 10 mM lidocaine also was significantly larger than the number that died in the presence of either 1 mM lidocaine or 30 mM choline (fig. 1A, $P < .05$). There was no statistically significant difference between either the number or the percentage of neurons that died in the presence of choline (at 30 or 100 mM) *versus* those that died in the presence of 1 mM lidocaine ($P > .05$).

In addition to neurons, DRG cultures contain several non-neuronal cells including Schwann cells and fibroblasts. Lidocaine did not have a statistically significant effect on the population of non-neuronal DRG cells. The number of trypan blue stained non-neuronal cells after exposure to 100 mM choline was 54 ± 13.9 ($n = 8$ coverslips), whereas that observed after exposure to 100 mM lidocaine was 87.8 ± 15.1 ($P > .05$) ($n = 8$ coverslips). Similarly, there was no significant difference between the percentage of non-neuronal cells stained with trypan blue after exposure to 100 mM choline

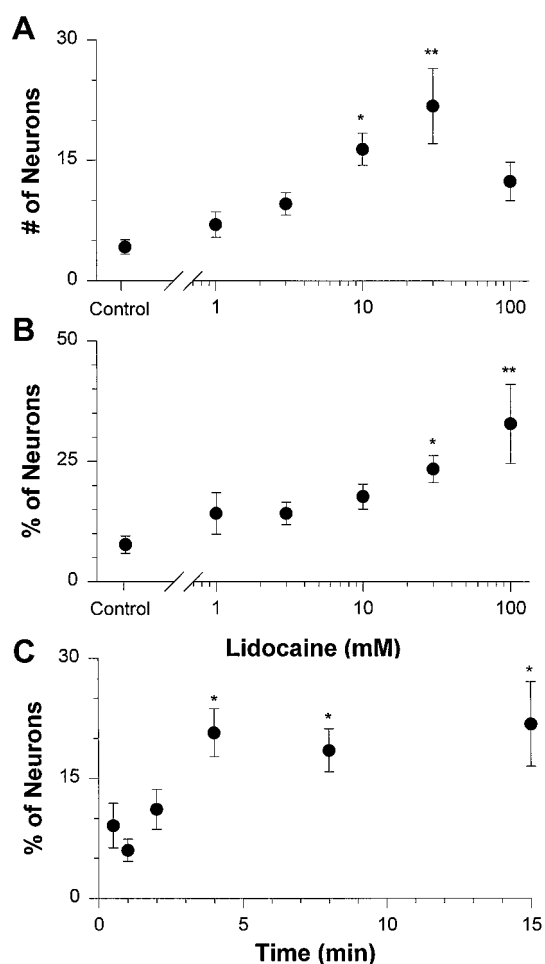


Fig. 1. Lidocaine induces cell death. (A) Lidocaine induces a dose-dependent increase in the number of "dead" (*i.e.*, trypan blue-stained) neurons remaining on coverslips 1 hr after a 15-min exposure to lidocaine. In this and subsequent figures * and ** reflect statistical probability (P) of $P < .05$ and $P < .01$, respectively. The number of dead neurons observed after exposure to 30 mM lidocaine was significantly (***) greater than that observed after exposure to either control solutions or 1 mM lidocaine. The number of dead neurons observed after exposure to 10 mM lidocaine was significantly (*) greater than that observed after exposure to control solutions or 1 mM lidocaine. (B) Data presented in A, analyzed as a percentage of the total number of neurons remaining on the coverslips; the percentage of dead neurons observed after exposure to 100 mM lidocaine was significantly (***) greater than that observed after exposure to either control solutions or 1 mM lidocaine. Similarly, the percentage of dead neurons observed after exposure to 30 mM lidocaine was significantly (*) greater than that observed after exposure to control solutions. The discrepancy between the two dose-response curves, particularly regarding the 100 mM dose of lidocaine, reflects the fact that the total number of neurons remaining attached to the coverslips decreased with increasing concentrations of lidocaine. Data are presented as mean \pm S.E.M. of cells counted on two coverslips from each of four different rats. (C) Time dependence of lidocaine-induced cell death. DRG neurons were exposed to 30 mM lidocaine for 0.5, 1, 2, 4, 8 or 15 min, and then washed for 1 hr as described in A. A significant increase in the percentage of dead neurons was detected after a 4-min exposure (*). Data are mean \pm S.E.M. from neurons counted on three coverslips from each of two different rats.

($10.6 \pm 2.6\%$) *versus* exposure to 100 mM lidocaine ($15 \pm 2.5\%$).

To investigate the time dependence of lidocaine-induced neurotoxicity, we determined the percentage of neurons stained with trypan blue after exposure to 30 mM lidocaine for 0.5, 1, 2, 4, 8 and 15 min. After the application of lidocaine, neurons were washed in normal bath solution for 1 hr.

A significant increase in the percentage of trypan blue-stained neurons was observed after a 4-min exposure to lidocaine (fig. 1C, $P < .05$). No significant increase in the percentage of trypan blue stained neurons was observed after exposure to lidocaine for less than 4 min.

Lidocaine depolarizes DRG neurons. Many neurotoxic agents are excitotoxins, *i.e.*, they damage neurons by causing excessive excitation. To determine whether lidocaine can exert direct effects on DRG neurons that might underlie an excitotoxic action, we tested the hypothesis that lidocaine depolarizes DRG neurons. We observed that lidocaine, at concentrations greater than 3 mM, depolarizes DRG neurons (fig. 2, A, B and C). This effect is dose-dependent, with an

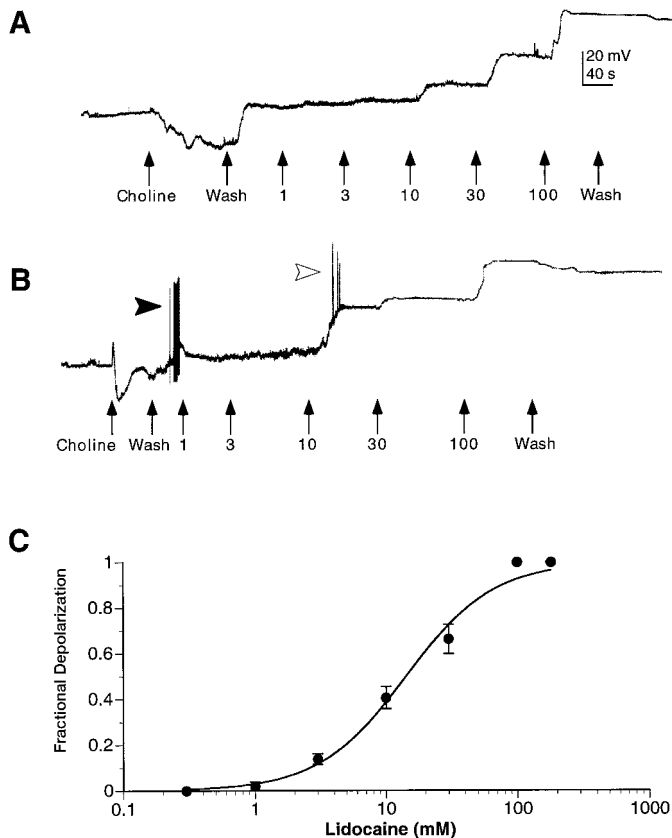


Fig. 2. Lidocaine depolarizes neurons. Whole-cell patch-clamp configuration was used to record from DRG neurons in current-clamp. Bath solution was perfused continuously at a rate of 2 ml/min. Test agents were applied through the perfusion system. (A) Changes in membrane potential observed in a 27- μ m diameter DRG neuron in response to choline (100 mM) and increasing concentrations of lidocaine (1, 3, 10, 30 and 100 mM). The resting potential in this neuron, recorded immediately after establishing whole-cell access, was -54 mV. After application of 100 mM lidocaine, the membrane potential in this neuron was $+6$ mV. (B) Changes in membrane potential observed in a 25- μ m diameter DRG neuron in response to the same experimental protocol applied to the neuron described in A. The resting potential in this neuron, recorded immediately after establishing whole-cell access, was -51 mV. After application of 100 mM lidocaine, the membrane potential in this neuron was $+2$ mV. A spontaneous burst of action potentials was observed after washout of 100 mM choline solution (filled arrowhead). Calcium spikes were evoked in the same neuron in response to the membrane depolarization induced by the application of 10 mM lidocaine (open arrowhead). (C) Pooled changes in membrane potential observed in DRG neurons in response to increasing concentrations of lidocaine ($n = 11$, except for the 60 mM concentration where $n = 5$). Data were collected as described for the neurons illustrated in A and B. Data were normalized by determining the fractional change in membrane potential as described under "Methods" and fitted with a Michaelis-Menten equation.

EC₅₀ of 14 mM (fig. 2C). The DRG neurons exhibited a maximally depolarized potential beyond which lidocaine could not drive the membrane potential, regardless of drug concentration or duration of application. This potential was $+3 \pm 2.1$ mV ($n = 16$). Neurons depolarized to near $+3$ mV by high concentrations (>30 mM) of lidocaine failed to repolarize within 10 min after washout of the lidocaine (observed in 11 of 11 neurons tested).

Although our standard electrophysiological protocol involves the study of only one neuron from each coverslip, on five different coverslips, we recorded from a second neuron subsequent to observing the irreversible depolarization of the first neuron with lidocaine. The mean resting potential (-54.6 ± 1.3 mV) of these five neurons previously exposed to 100 mM lidocaine was not significantly different from the resting potential (-54 ± 2.3 mV) of "naïve" neurons (*i.e.*, those never exposed to lidocaine, $P > .05$). In addition, it was possible to evoke a "normal" action potential from each of these five neurons. Furthermore, each of these five neurons subsequently was depolarized irreversibly by the application of 100 mM lidocaine. These observations suggest that the apparent irreversibility of the lidocaine-induced depolarization might be due to an interaction between lidocaine-induced effects and changes in the neuron caused by dialysis of the cytoplasm by the whole-cell patch-clamp electrode.

Lidocaine induces block of voltage-gated Na⁺ current and the somatic action potential. Our observation of an excitatory effect of lidocaine on DRG neurons contrasts with the conventional view of local anesthetics as inhibitory agents that act primarily to reduce voltage-gated sodium currents. To compare the excitatory and inhibitory actions of this local anesthetic, we performed voltage-clamp recordings to measure quantitatively the effect of lidocaine on the two principal voltage-gated sodium currents in DRG neurons (fig. 3A). TTX-S I_{Na} is a rapidly activating and inactivating low-threshold current blocked by nanomolar concentrations of tetrodotoxin, whereas TTX-R I_{Na} is a more slowly activating and inactivating high-threshold current resistant to micromolar concentrations of TTX. Currents were evoked from a potential of -100 mV to remove steady-state inactivation (fig. 3A; Gold *et al.*, 1996b). Both TTX-S I_{Na} and TTX-R I_{Na} were blocked by lidocaine in a dose-dependent fashion (fig. 3, B and C) with half-maximal concentrations equal to 41 and 101 μ M, respectively (fig. 3C). These results are similar to previously reported values (Roy and Narahashi, 1992). The application of 1 mM lidocaine, a concentration that completely blocked voltage-gated Na⁺ current also completely blocked the somatic action potential (fig. 3C, insert). The effects of 1 mM lidocaine on voltage-gated sodium currents and somatic action potentials were fully and rapidly reversible (fig. 3B). The concentration of lidocaine required to completely block voltage-gated Na⁺ currents (1 mM) is more than an order of magnitude lower than the concentration at which we begin to see lidocaine-induced cell death.

Lidocaine stimulates Ca⁺⁺ influx and mobilization of internal stores in DRG neurons. Because DRG neurons were depolarized by lidocaine at concentrations similar to those at which we observed lidocaine-induced cell death, and because Ca⁺⁺-dependent mechanisms contribute to excitotoxic cell death in a variety of neurotoxic models (Orrenius and Nicotera, 1994), it is possible that an increase in intracellular Ca⁺⁺ concentration ($[Ca^{++}]_i$) contributes to lido-

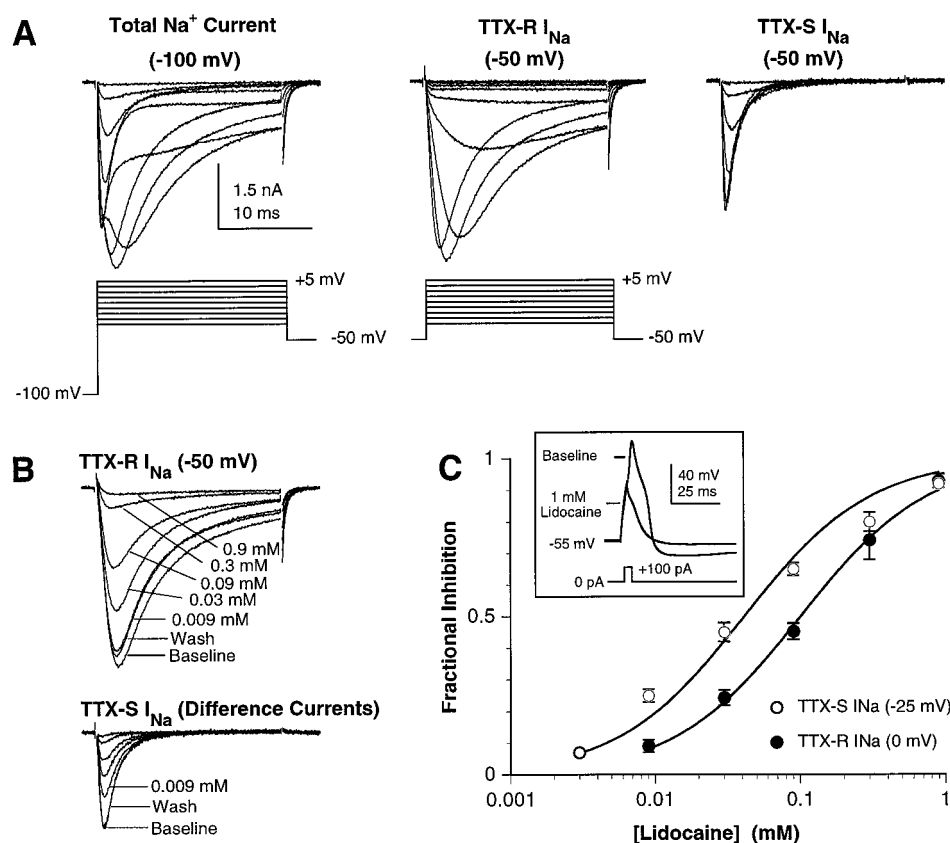


Fig. 3. Lidocaine inhibits voltage-gated sodium currents in DRG neurons. Two voltage-gated sodium currents (I_{Na}) in DRG neurons are distinguished by sensitivity to TTX and gating properties. (A, left panel) Voltage-gated sodium current evoked from a 27- μ m DRG neuron during 15 msec command potentials ranging from -35 to $+5$ mV, after a 500-msec prepulse to -100 mV; the holding potential was -70 mV. The voltage-clamp protocol used to evoke the currents is shown beneath the current traces. (A, middle panel) TTX-R I_{Na} was isolated by changing the prepulse amplitude to -50 mV, a potential at which TTX-S I_{Na} is inactivated. (A, right panel) TTX-S I_{Na} was isolated as the difference between the current evoked from -100 mV and the current evoked from -50 mV. (Currents evoked at -5 , 0 and $+5$ mV have been omitted for clarity.) (B) Dose-dependent inhibition of TTX-R I_{Na} (top) and TTX-S I_{Na} (bottom). TTX-R I_{Na} was isolated as described in A; current traces evoked at 0 mV 30 sec after the application of each concentration of lidocaine are plotted. TTX-S I_{Na} was isolated as described in A; current traces were evoked at -25 mV 30 sec after the application of each concentration of lidocaine. The same series of lidocaine concentrations were tested on TTX-S I_{Na} as were tested on TTX-R I_{Na} ; the lidocaine concentration labels have been omitted for clarity. (C) Pooled dose-response relationships for TTX-R I_{Na} and TTX-S I_{Na} ($n = 7$). To facilitate pooling of data, the response of each cell to lidocaine was normalized by deriving the fractional inhibition of current observed at each concentration of lidocaine as described under "Methods." Data were fitted with a Michaelis-Menten equation as described under "Methods." (Inset) Membrane depolarization evoked in response to a 2-msec depolarizing current injection, before (Baseline) and after (1 mM Lidocaine) the application of 1 mM lidocaine. The current injected (100 pA) was 5 times the minimal current required to evoke an action potential in this neuron. The overshooting action potential was blocked completely by lidocaine. Voltage- and current-clamp data were sampled at 50 KHz and filtered at 5 KHz.

caine-induced cell death. Furthermore, it is possible that the source of the increase in $[Ca^{++}]_i$ was extracellular Ca^{++} possibly entering neurons *via* voltage-gated Ca^{++} channels activated by the lidocaine-induced depolarization of the plasma membrane. To investigate these possibilities we determined whether lidocaine-induced cell death could be diminished by exposing DRG cells to 30 mM lidocaine in the presence of nominally Ca^{++} -free bath solution. No significant increase in cell survival was observed when lidocaine was applied in the absence of extracellular Ca^{++} (data not shown).

Evidence shows that local anesthetics block a sarcoplasmic reticulum Ca^{++} -ATPase (Karon *et al.*, 1995; Kutchai *et al.*, 1994), and therefore, also may block an endoplasmic reticulum Ca^{++} -ATPase, possibly resulting in an increase of $[Ca^{++}]_i$ *via* increased efflux of Ca^{++} from intracellular stores. Therefore, we hypothesized that lidocaine toxicity might result from lidocaine-induced release of Ca^{++} from intracellular stores. To test this hypothesis, we used the Ca^{++} -sensitive dye, Fura-2, to determine whether lidocaine

induces an increase in $[Ca^{++}]_i$ *via* release from intracellular stores.

Lidocaine dose-dependently increased $[Ca^{++}]_i$ in neurons (fig. 4). Examples of the changes in $[Ca^{++}]_i$ induced by brief (30-sec) applications of increasing concentrations of lidocaine in two different neurons is shown in figure 4A. As suggested by the examples presented in figure 4A, there was variability among DRG neurons in the sensitivity to lidocaine with respect to the change in $[Ca^{++}]_i$. This variability is demonstrated clearly by plotting the fraction of neurons responding to lidocaine with an increase in $[Ca^{++}]_i$ *versus* lidocaine concentration (fig. 4B). A neuron was considered responsive to lidocaine if the change in $[Ca^{++}]_i$ 30 sec after the application of lidocaine was more than two standard deviations larger than the mean change in $[Ca^{++}]_i$ observed during the same period in the absence of lidocaine. More than 50% of DRG neurons are responsive to the application of 10 mM lidocaine (fig. 4B).

To estimate the dose dependence of the lidocaine-induced change in $[Ca^{++}]_i$, data from 40 neurons were normalized

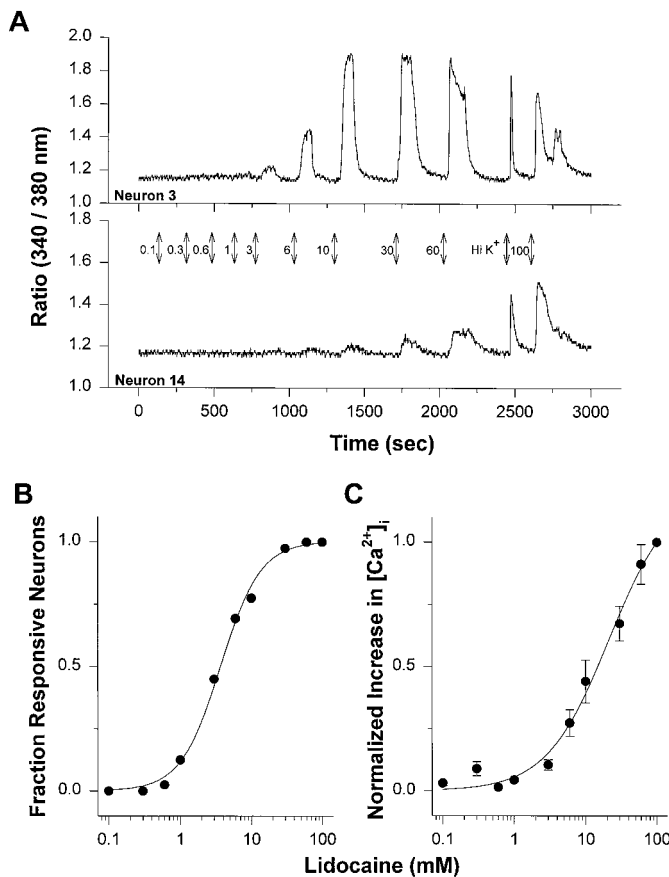


Fig. 4. Lidocaine increases the concentration of intracellular Ca^{++} in DRG neurons. (A) Changes in Fura-2 fluorescence in response to increasing concentrations of lidocaine recorded in two different DRG neurons on the same coverslip. Increasing concentrations of lidocaine (mM) were applied for 30 sec each *via* the bath perfusion system at the times indicated by the arrows. To assess whether a depolarization-induced increase in $[\text{Ca}^{++}]_i$ could be evoked in DRG neurons, the response to a 5-sec application of 50 mM K^+ (Hi K^+) either was assessed before (as shown) or after application of 100 mM lidocaine. (B) There was a dose-dependent increase in the fraction of neurons responding to lidocaine with an increase in $[\text{Ca}^{++}]_i$. A neuron was considered responsive to a given concentration of lidocaine if the increase in $[\text{Ca}^{++}]_i$ in response to lidocaine was more than two standard deviations above base line; all 41 neurons tested were responsive to 60 mM lidocaine. (C) Pooled dose-response relationship for lidocaine-induced increase in $[\text{Ca}^{++}]_i$ ($n = 41$). To facilitate pooling of data, the response of each neuron was normalized with respect to the peak increase in $[\text{Ca}^{++}]_i$ evoked in response to 100 mM lidocaine. Data were fitted with a Michaelis-Menten equation as described under "Methods."

with respect to the change induced by the application of 100 mM lidocaine, pooled and fitted with a Michaelis-Menten equation (fig. 4C). The fitted EC_{50} value for these data was 21 mM and the Hill coefficient was 1. These results suggest that the lidocaine-induced increase in $[\text{Ca}^{++}]_i$ was not saturated at 100 mM lidocaine (fig. 4C).

To determine whether the lidocaine-induced increase in $[\text{Ca}^{++}]_i$ was the result of Ca^{++} entry through the plasma membrane, we determined the effect of lidocaine on $[\text{Ca}^{++}]_i$ in the presence of a nominally Ca^{++} -free bath solution. Although the increase in $[\text{Ca}^{++}]_i$ induced by the bath application of 50 mM K^+ was eliminated completely in the presence of nominally Ca^{++} -free bath solution, the increase in $[\text{Ca}^{++}]_i$ induced by 30 mM lidocaine was reduced only partially (fig. 5). The magnitude of the reduction varied among neurons; the mean response to 30 mM lidocaine in Ca^{++} -free bath as

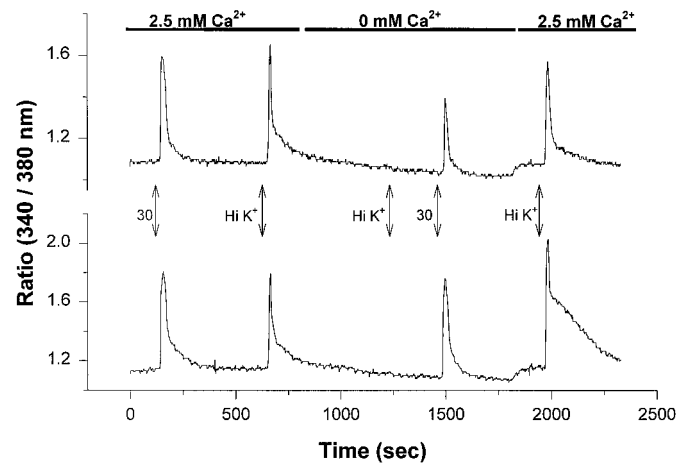


Fig. 5. Lidocaine increases $[\text{Ca}^{++}]_i$ in DRG neurons *via* influx through the plasma membrane as well as release from internal stores. Changes in Fura-2 fluorescence were recorded in DRG neurons in response to 30 mM lidocaine and to 50 mM potassium (Hi K^+) in the presence and absence of Ca^{++} in the bath solution. Both 30 mM lidocaine and 50 mM potassium evoked an increase in $[\text{Ca}^{++}]_i$ in the presence of 2.5 mM Ca^{++} in the bath solution. The response to 50 mM potassium was eliminated completely in nominally Ca^{++} -free bath solution (0 mM Ca^{++}), whereas the response to 30 mM lidocaine was retained. The extent to which the response to 30 mM lidocaine was decreased in nominally Ca^{++} -free bath solution varied between neurons as illustrated by the different responses of the two neurons shown.

a percentage of the response to 30 mM lidocaine in 2.5 mM Ca^{++} bath was $69.1 \pm 6.1\%$, $n = 13$; range, 20.2 to 93.0%. These data suggest that the increase in $[\text{Ca}^{++}]_i$ in neuronal cells reflects the release of Ca^{++} from internal stores in addition to influx of extracellular Ca^{++} . Further evidence supporting the suggestion that the lidocaine-induced increase in $[\text{Ca}^{++}]_i$ partly reflects the release of Ca^{++} from internal stores is provided by observations which indicate that lidocaine-sensitive Ca^{++} stores can be depleted and refilled. That is, the lidocaine-induced increase in $[\text{Ca}^{++}]_i$ diminishes with repeated applications of lidocaine in Ca^{++} -free bath solution, but recovered in bath solution containing Ca^{++} (data not shown). In control experiments, choline solution (30 mM) iso-osmotic with the lidocaine solution, had no effect on $[\text{Ca}^{++}]_i$ ($n = 8$, data not shown).

A 15-min application of lidocaine (30 mM) that mimicked the lidocaine application in the foregoing cell death experiments induced a rapid increase in $[\text{Ca}^{++}]_i$ that recovered to near base-line levels during the 15-min application period (fig. 6). Washout of lidocaine induced another transient increase in $[\text{Ca}^{++}]_i$ that subsequently recovered to base line (fig 6, top panel). The 15-min application of 30 mM lidocaine caused apparent lysis of several neurons, indicated by a complete loss of the Fura-2 fluorescence from these cells (fig. 6, bottom panel); these neurons did not remain attached to the coverslip. After a 1-hr wash with normal bath solution, application of 50 mM K^+ failed to induce an increase in $[\text{Ca}^{++}]_i$ in 42% ($n = 12$) of neurons that were not lysed, which indicates that the excitability of neurons was impaired by the 15-min application of lidocaine. A 15-min application of iso-osmotic choline (30 mM) had no effect on $[\text{Ca}^{++}]_i$ or the response to 50 mM K^+ (data not shown).

Increased $[\text{Ca}^{++}]_i$ contributes to lidocaine-induced neurotoxicity. To further investigate the contribution of the lidocaine-induced increase in $[\text{Ca}^{++}]_i$ to lidocaine-induced

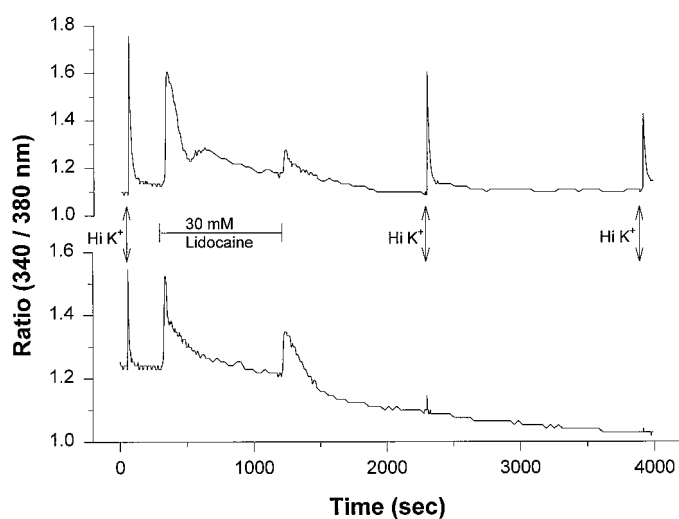


Fig. 6. Prolonged application of lidocaine induces a transient increase in $[Ca^{++}]_i$ in DRG neurons. A 15-min application of 30 mM lidocaine evoked a rapid increase in $[Ca^{++}]_i$ that decayed during a period of 15 min. Washout of lidocaine was associated with a transient increase in $[Ca^{++}]_i$. Some neurons were able to respond to a 5-sec application 50 mM potassium (Hi K^+) after a 15-min exposure to 30 mM lidocaine, with a transient increase in $[Ca^{++}]_i$ (top trace). In contrast, other neurons eventually failed to respond to 50 mM potassium (bottom trace). The apparent decrease in resting $[Ca^{++}]_i$ that developed after washout of lidocaine from the neuron represented in the bottom trace reflects the fact that the neuron failed to retain the Fura-2 dye after apparent lysis of its plasma membrane. This neuron eventually detached from the coverslip.

neurotoxicity, lidocaine was applied in the presence of nominally Ca^{++} -free bath solution (table 1, BS 3) to DRG neurons preloaded with the Ca^{++} buffer, BAPTA (see "Methods"). To confirm that the BAPTA loading protocol resulted in an intracellular concentration of BAPTA sufficient to inhibit the lidocaine-induced increase in $[Ca^{++}]_i$, the change in $[Ca^{++}]_i$ was measured in response to 30 mM lidocaine. In neurons ($n = 19$) preloaded with BAPTA, 30 mM lidocaine induced minimal change in $[Ca^{++}]_i$ (fig. 7A).

The effect of BAPTA on cell survival was assessed in a separate experiment. As shown in figure 7B, preloading cells with BAPTA significantly reduced lidocaine-induced death of DRG neurons; the percentage of neurons that failed to exclude trypan blue after exposure to 30 mM lidocaine was significantly lower than when cells underwent the loading procedure with DMSO alone ($P < .01$). Furthermore, the reduced level of trypan blue staining in BAPTA-loaded neurons was not significantly different from that in control experiments in which BAPTA-loaded cells were exposed to 30 mM choline ($P > .05$). The level of trypan blue staining in those control experiments, however, was greater than when neurons were not loaded with BAPTA nor exposed to Ca^{++} -free bath (e.g., fig. 1B), which suggests that the prolonged depression of $[Ca^{++}]_i$ induced by these manipulations itself causes some additional neurotoxicity.

Discussion

Our results indicate that application of lidocaine to DRG neurons at concentrations greater than 10 mM causes neuronal death. At a concentration of 30 mM, a 4-min application of lidocaine is sufficient to induce neuronal death. Concentrations of lidocaine inducing neuronal death are more than an order of magnitude greater than those required for com-

plete and reversible blockade of voltage-gated sodium channels and action potential generation. Lidocaine, at concentrations that cause neuronal death, also causes rapid depolarization of the neuronal membrane as well as increase in $[Ca^{++}]_i$ that apparently results from Ca^{++} influx through the plasma membrane in addition to Ca^{++} release from intracellular stores. Buffering the lidocaine-induced increase in $[Ca^{++}]_i$ significantly attenuates lidocaine-induced neuronal death.

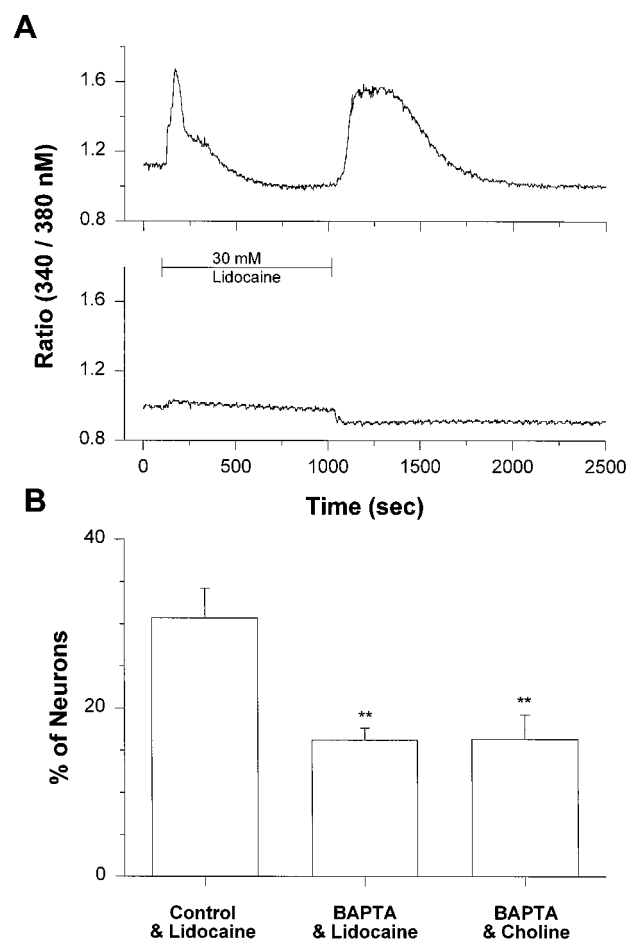


Fig. 7. Preventing the lidocaine-induced increase in $[Ca^{++}]_i$ inhibits lidocaine-induced neurotoxicity. The lidocaine-induced increase in $[Ca^{++}]_i$ was prevented by preloading neurons with BAPTA and applying lidocaine in the presence of nominally Ca^{++} -free bath solution. Neurons were preloaded with BAPTA as described under "Methods." (A) Examples of the effects of lidocaine (30 mM Lidocaine) on $[Ca^{++}]_i$ in neurons preincubated with the BAPTA vehicle, DMSO (top panel) or BAPTA (bottom panel). Both neurons were bathed in nominally Ca^{++} -free bath solution, and lidocaine was applied at the time indicated by the bar. The lidocaine-induced change in $[Ca^{++}]_i$ observed in the DMSO-loaded neuron was typical of the response observed in 30 other neurons. Similarly, the lidocaine-induced change in $[Ca^{++}]_i$ observed in the BAPTA-loaded neuron was typical of the response observed in 18 other neurons preloaded with BAPTA. (B) The mean percentage of neurons that were stained with trypan blue 1 hr after a 15-min application of 30 mM lidocaine was significantly (***) less in neurons preloaded with BAPTA (BAPTA & Lidocaine) than that in DMSO-treated control neurons (Control & Lidocaine). There was no significant difference between the mean percentage of neurons stained with trypan blue in the BAPTA-lidocaine group and that of neurons preloaded with BAPTA and exposed to 30 mM choline (BAPTA & Choline). Data for Control & Lidocaine and BAPTA & Lidocaine groups were obtained from neurons counted on five and eight coverslips, respectively, from two different rats; data for the BAPTA & Choline group were obtained from neurons counted on three coverslips from one rat.

Our measurement of lidocaine-induced toxicity likely underestimated the extent of lidocaine-induced cell death because of three factors. First, we only counted neurons that remained attached to the coverslip. One hour after a 15-min exposure to 100 mM lidocaine, the number of neurons remaining on the coverslips was reduced markedly. This loss of neurons likely reflects an increase in cell death rather than a nontoxic lidocaine-induced disruption of cell adhesion, as suggested by our observations during the Ca^{++} imaging experiments. Loss of neurons from the coverslip was associated with the lysis of the neurons. Second, we performed cell counts relatively soon after exposure to lidocaine; when counts were performed 24 hours after lidocaine exposure, there was almost a complete loss of neurons from the coverslips (data not shown). Third, failure to exclude trypan blue represents rather severe damage to neurons; assays for metabolic processes might have revealed a more widespread but less severe neuronal injury as opposed to death. In future studies, such assays may be particularly useful for identifying underlying mechanisms of toxicity.

Many of our observations support the suggestion that lidocaine-induced toxicity is the result of a direct action of the compound itself, rather than the vehicle or the method of administration. First, because crystalline lidocaine was dissolved directly in HEPES-buffered bath solution in which the pH was adjusted to 7.2, toxicity associated with the acidic nature of a nonbuffered solution was avoided. Second, the high osmolality of a 100 mM lidocaine solution was not the cause of neuronal death, because cell death was not caused by exposure to bath solution containing 100 mM choline. Third, because we performed all experiments in continuously perfused chambers, it is unlikely that cell death was an indirect effect that occurred secondarily to a lidocaine-induced release of cytotoxic compounds from neuronal or non-neuronal cells: any such compounds would be diluted rapidly and removed by the flowing bath solution. Furthermore, when studied within 24 hr of plating (as in the present experiments) the vast majority of DRG neurons in this preparation do not contact each other or non-neuronal cells, thereby avoiding the possibility of direct interactions among the cells.

Our results indicate that lidocaine toxicity is not the result of an immediate and irreversible breakdown in the integrity of the plasma membrane, as would be reflected by a rapid and permanent loss in membrane potential. The mechanism of the lidocaine-induced depolarization currently is unknown, but may reflect the simultaneous blockade of ion channels and pumps responsible for the maintenance of neuronal resting potential; lidocaine-induced depolarization has been reported previously by other investigators (Lambert *et al.*, 1994). Although the axonal membrane repolarizes after wash of lidocaine (Lambert *et al.*, 1994), an irreversible conduction block was observed in axons after 3 min exposure of the axon to lidocaine at concentrations as low as 40 mM (Bainton and Strichartz, 1994). That we observed recovery of somal action potential after exposure of DRG neurons to even higher concentrations of lidocaine raises the possibility that different mechanisms underlie neuronal death and loss of conduction. However, because an increase in $[\text{Ca}^{++}]_i$ can disrupt cytoskeleton (Neely and Gesemann, 1994; Uto *et al.*, 1994; Waxman *et al.*, 1993), and disruption of cytoskeleton can decrease excitability (Sakai *et al.*, 1985; Waxman *et al.*,

1993), a lidocaine-induced increase in $[\text{Ca}^{++}]_i$ possibly could contribute to both cell death (see below) and long-lasting conduction failure.

Lidocaine reportedly blocks high threshold voltage-gated Ca^{++} channels in DRG neurons with an IC_{50} of ≈ 2.8 mM (Sugiyama and Muteki, 1994). Therefore, these channels should be blocked by concentrations of lidocaine that cause sufficient depolarization to activate them. Whether lidocaine induces Ca^{++} entry into DRG neurons *via* low-threshold, voltage-gated Ca^{++} or *via* other Ca^{++} -permeable channels has yet to be determined.

The release of Ca^{++} from internal stores may reflect the inhibition of a Ca^{++} -ATPase of endoplasmic reticulum, as lidocaine has been shown to block a Ca^{++} -ATPase of the sarcoplasmic reticulum (Karon *et al.*, 1995; Kutchai *et al.*, 1994). Similar to the effects of Ca^{++} -ATPase inhibitors such as thapsigargin, the lidocaine-induced increase in $[\text{Ca}^{++}]_i$ is transient, in some neurons decaying to base line within 15 min, (presumably reflecting a depletion of internal Ca^{++} stores), and is followed by a transient increase in $[\text{Ca}^{++}]_i$, after wash of lidocaine [presumably reflecting "capacitative Ca^{++} entry" that may be associated with the refilling of internal Ca^{++} stores (Clapham, 1995)]. Although it also is possible that lidocaine uncouples the mitochondrial electrochemical gradient to release stored Ca^{++} , it previously has been demonstrated that, under resting conditions, mitochondria in DRG neurons store very little releasable Ca^{++} (Werth and Thayer, 1994).

Our observation that preventing the lidocaine-induced increase in $[\text{Ca}^{++}]_i$ significantly attenuates lidocaine-induced neurotoxicity strongly suggests that the increase in $[\text{Ca}^{++}]_i$ is an underlying mechanism of toxicity. This suggestion is consistent with the observation made by Bainton and Strichartz (1994) that L-type Ca^{++} channel blockers made nerves more resistant to the toxic effects of lidocaine. However, we cannot exclude the possibility that the apparent decrease in base-line $[\text{Ca}^{++}]_i$ in BAPTA-loaded neurons exposed to Ca^{++} -free bath (see fig. 7A) also may have contributed to the decrease in lidocaine-induced neurotoxicity.

Consistent with the delayed nature of Ca^{++} -dependent neurotoxicity, for most neurons lidocaine-induced death was not evident during, or immediately after, application of lidocaine. For example, after washout of lidocaine, the membrane potential returned to the original resting potential, and 50 mM K^+ was able to evoke an increase in $[\text{Ca}^{++}]_i$ in six of seven neurons 15 min after wash of the lidocaine. In addition, the lidocaine-induced increase in $[\text{Ca}^{++}]_i$ returns to resting level after wash of lidocaine. Our results suggest that a 4-min increase in $[\text{Ca}^{++}]_i$ is sufficient to induce cell death. That an increase in $[\text{Ca}^{++}]_i$ as short as 5 min is sufficient to induce delayed neuronal death has been well documented (Manev *et al.*, 1991; Randall and Thayer, 1992). Although the cellular processes engaged by the lidocaine-induced increase in $[\text{Ca}^{++}]_i$ that result in cell death have yet to be identified, there is evidence that capsaicin-induced delayed death of DRG neurons depends on the activation of Ca^{++} -dependent proteases (Chard *et al.*, 1995).

The incidence of nerve injury resulting from the clinical use of lidocaine is relatively low, possibly because the concentration of lidocaine at the neuronal membrane rarely reaches levels necessary to induce injury. With the usual peripheral routes of administration there are large barriers

to diffusion (Ritchie *et al.*, 1965). Consequently, intraneuronal lidocaine concentration is only 1.6% of the injected concentration at full block (Popitz-Bergez *et al.*, 1995).

Dilution of the local anesthetic in the relatively large volume of the subarachnoid space is a major factor contributing to the minimization of local anesthetic concentrations at the neuronal membrane after spinal administration (Rigler *et al.*, 1991). Importantly, diffusion barriers are considerably lower for spinal administration of anesthetics compared with those for peripheral administration, as suggested by the observation that lidocaine has an EC_{50} of $\approx 230 \mu\text{M}$ for inhibition of evoked action potentials in isolated dorsal roots (Jaffe and Rowe, 1996). Furthermore, spinally administered local anesthetics often are distributed nonhomogeneously (Drasner *et al.*, 1994; Robinson *et al.*, 1994). The maldistribution of anesthetic is a potential problem that may be exacerbated with the use of hyperbaric solutions specifically designed to control the distribution of spinally administered local anesthetics. Therefore, given our results indicating that brief exposure of neurons to relatively low concentrations of lidocaine may result in neurotoxicity, it is not unreasonable to suggest that neurotoxic levels of lidocaine may be approached clinically with routine spinal administration of this local anesthetic. Indeed, it has been reported that, 5 min after administration of a 5% solution of lidocaine, the mean cerebrospinal fluid concentration was close to 16 mM (Van Zundert *et al.*, 1996). Thus, while diffusion and dilution may limit the concentration of lidocaine at the neuronal membrane, and therefore minimize the clinical sequela resulting from the use of lidocaine, our results begin to define an upper limit on the therapeutic window governing the use of lidocaine; under conditions in which barriers to diffusion have been removed or dilution is limited, lidocaine may become neurotoxic.

Summary and conclusions. We have demonstrated for the first time that lidocaine can act directly on mammalian sensory neurons to cause membrane depolarization, an increase in $[\text{Ca}^{++}]_i$, and neurotoxicity at doses relevant to the clinical use of lidocaine. Importantly, we have provided evidence indicating that the lidocaine-induced increase in $[\text{Ca}^{++}]_i$ is an underlying mechanism of lidocaine-induced neurotoxicity. Better understanding of the mechanisms of toxicity might aid in the development of pharmacological strategies to avoid the neurologic injury that complicates the use of spinally administered local anesthetics.

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