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Increase in intracellular Ca^{2+} concentration is not the only cause of lidocaine-induced cell damage in the cultured neurons of *Lymnaea stagnalis*

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Abstract
Purpose. To determine whether the increase in intracellular Ca^{2+} concentration induced by lidocaine produces neurotoxicity, we compared morphological changes and Ca^{2+} concentrations, using fura-2 imaging, in the cultured neurons of *Lymnaea stagnalis*.
Methods. We used BAPTA-AM, a Ca^{2+} chelator, to prevent the increase in the intracellular Ca^{2+} concentration, and Calcimycin A23187, a Ca^{2+} ionophore, to identify the relationship between increased intracellular Ca^{2+} concentrations and neuronal damage without lidocaine. Morphological changes were confirmed using trypan blue to stain the cells.
Results. Increasing the dose of lidocaine increased the intracellular Ca^{2+} concentration; however, there was no morphological damage to the cells in lidocaine at 3×10^{-3} M. Lidocaine at 3×10^{-2} M increased the intracellular Ca^{2+} concentration in both saline (from 238 ± 63 to 1038 ± 156 nM) and Ca^{2+}-free medium (from 211 ± 97 to 1046 ± 169 nM) and produced morphological damage and shrinkage, with the formation of a rugged surface. With the addition of BAPTA-AM, lidocaine at 3×10^{-2} M moderately increased the intracellular Ca^{2+} concentration (from 150 ± 97 to 428 ± 246 nM) and produced morphological damage. These morphologically changed cells were stained dark blue with trypan blue dye. The Ca^{2+} ionophore increased the intracellular Ca^{2+} concentration (from 277 ± 191 to 1323 ± 67 nM) and decreased it to 186 ± 109 nM at 60 min. Morphological damage was not observed during the 60 min, but became apparent a few hours later.
Conclusion. These results indicated that the increase in intracellular Ca^{2+} concentration is not the only cause of lidocaine-induced cell damage.

Key words Lidocaine · Neurotoxicity · Ca^{2+} concentration

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Introduction

Clinical profiles of neurotoxicity caused by local anesthetics have been based on many reports of cauda equina or transient neurologic symptoms following spinal anesthesia [1,2]. The mechanism of local anesthetic neurotoxicity has not been clearly demonstrated. One of the possible mechanisms is an extreme increase in intracellular Ca^{2+} concentration induced by lidocaine [3,4]. Another suggestion regarding lidocaine-induced neurotoxicity is that lidocaine directly disrupts the nerve membrane and produces nerve injury [5,6]. These types of neurotoxicity are judged by the leakage of enzymes from the cytoplasm or by disruption of a model membrane.

We previously reported the neurotoxicity of local anesthetics to growth cones and neurites [7]. To clarify the mechanism of local anesthetic neurotoxicity, we compared the relationship between morphological changes and elevated Ca^{2+} concentrations in cells. Local anesthetics increase the intracellular Ca^{2+} concentration and modulate neuronal calcium signaling through multiple sites of action [8]. In the present experiments, morphological changes in cells, such as shrinkage or the formation of rugged surfaces, were considered toxic to the cells, and were analyzed in relation to the intracellular Ca^{2+} concentration. This cell damage was further confirmed using trypan blue. To determine the effects of Ca^{2+} concentration outside the cells, we compared them in saline and in a Ca^{2+}-free medium. The neuronal Ca^{2+} concentrations in a cell were changed using a Ca^{2+} chelator, BAPTA-AM, to decrease the effects of lidocaine on the Ca^{2+} outflow inside the cell, as well as to reduce the neurotoxicity. Furthermore, we used a Ca^{2+} ionophore to identify the relationship between the elevated Ca^{2+} concentration without lidocaine and neuronal lysis, using fura-2 imaging in cultured neurons of *Lymnaea stagnalis*.

Methods

Cell culture

Laboratory-raised stocks of the freshwater snail, *Lymnaea stagnalis*, at 3 to 6 months of age, with a shell length of 10–25 mm, were used. Lymnaea normal saline contained 51.3 mM NaCl, 1.7 mM KCl, 4.1 mM $CaCl_2$, 1.5 mM $MgCl_2$, and 5.0 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), and the pH was adjusted to 7.9 with 1 M NaOH. Antibiotic normal saline contained gentamicin at 150 $\mu g \cdot ml^{-1}$ (G-3632; Sigma, St. Louis, MO, USA) as the sole antibiotic. The defined growth medium consisted of serum-free, 50% Liebowitz L-15 medium (GIBCO, Grand Island, NY, USA) with inorganic salts and 20 $\mu g \cdot ml^{-1}$ of gentamicin. Inorganic salts in the medium included 40 mM NaCl, 1.7 mM KCl, 4.1 mM $CaCl_2$, 1.5 mM $MgCl_2$, and 10 mM HEPES, and the pH was adjusted to 7.9. After de-shelling, snails were transferred to antibiotic normal saline in a sterile dissection dish. The central ganglionic rings were isolated, using a standard dissection procedure [9], and then pinned to the silicone rubber base of a tissue culture plate. Ganglia were treated with trypsin (Sigma) at 2 $mg \cdot ml^{+}$ in the defined medium for 25 min. Subsequently, ganglia were treated with a soybean trypsin inhibitor (Sigma) at 2 $mg \cdot ml^{-1}$ in the defined medium for 10 min. Before the removal of identified cultured neurons (RPD1, RPeD1, and A cluster) [9], the innerconnective tissue sheath was dissected, with fine forceps, from the ganglia. Neurons were transferred to poly-L-lysine-coated culture glass-bottomed microwell dishes (Mat Tec, Ashland, MA, USA) with 3 ml of conditioned medium by gentle suction with a siliconized (Sigmacoate; Sigma) microforge finepolished pipette that had an outside diameter of 1.5 mm (IB-150F; WPI, Sarasota, FL, USA). The conditioned medium was prepared to incubate the ganglion in the defined medium (one ganglion per ml) for a few days [10]. After 16–20 h of cell transfer, evidence of nerve growth was observed under an optical microscope. The changes in cultured neurons were observed using a color video camera (XC-003; SONY, Tokyo, Japan) mounted directly on an inverted microscope (Axiovert S100; Zeiss, Göttingen, Germany), and images were recorded using a digital videotape recorder (DVCAM; SONY).

After recording baseline morphological pictures of the cell body, high concentrations (1×10^{-3}, 1×10^{-2}, 1×10^{-1}, and 1 M) of lidocaine (Sigma) were gently added to give final concentrations ranging from 3×10^{-5} to 3×10^{-2} M of lidocaine. Lidocaine was prepared in highly purified distilled water. The volume of added local anesthetic was less than 1/30 of the volume of media. Cultured neurons were examined 5 min after exposure to each concentration of local anesthetic. Each cell was assigned a morphological change score (0–2): 0, no change, similar to baseline features; 1, moderate change, shrunken cell body with a smooth surface; and 2, severe change, shrunken cell with a rugged surface (Fig. 1).

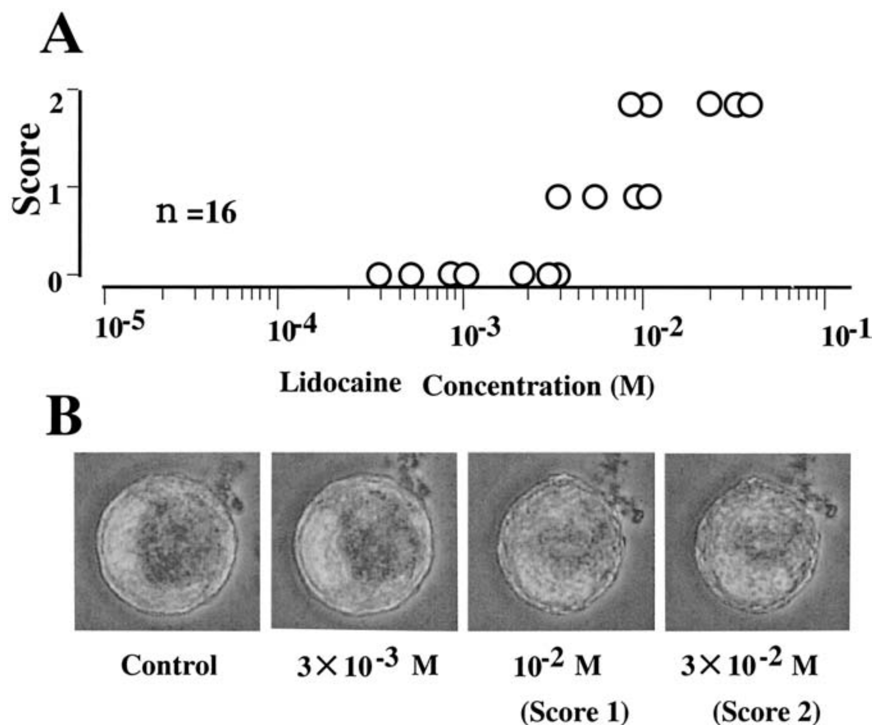


Fig. 1A,B. Relationship between the dose of lidocaine and morphological damage in cultured neurons. Each cell was assigned a morphologic change score (0–2): 0, no change, similar to baseline features; 1, moderate change, shrunken cell body with a smooth surface; and 2, severe change, shrunken cell with a rugged surface

Measurements of the Ca^{2+} concentration

For the measurements of the Ca^{2+} concentration, the fluorescent indicator, acetoxymethyl ester of fura-2 (fura-2/AM) was used. Fura-2/AM (Dojindo, Tokyo, Japan) in dimethyl sulfoxide (DMSO) was mixed with saline and added to the conditioned medium containing a neuron, at a final concentration of $10 \mu M \cdot ml^{-1}$. After incubation at 20°C for 45 min, this conditioned medium was removed, and the neuron was suspended in normal saline for 1 h. Normal saline was gently exchanged for a Ca^{2+}-free medium (51.3 mM NaCl, 1.7 mM KCl, 1.5 mM $MgCl_2$, 5 mM HEPES, and 5 mM ethyleneglycoltetraacetic acid [EGTA], with the pH adjusted to 7.9) in which $CaCl_2$ was exchanged for EGTA. The intensity of intracellular fura-2 fluorescence was measured at two quickly alternating excitation wavelengths (340/380 nm) and continuously recorded at 510 nm with an inverted fluorescent microscope (TE-300; Nikon, Tokyo, Japan), a cooled high-speed charge-coupled device (CCD) video camera (C-6970; Hamamatu Photonics, Tokyo, Japan), and a calcium imaging system (Argus-Hisca; Hamamatu Photonics). Background fluorescent images were subtracted before analysis. Two fluorescence ratios were converted into Ca^{2+} concentrations, using a calibration curve made using a calibration kit (Fura-2 Calcium Imaging Calibration Kit; Molecular Probes, Eugene, OR, USA).

To clarify the effects of the extracellular Ca^{2+} concentration, normal saline was gently exchanged for the Ca^{2+}-free medium. Then, the Ca^{2+} chelator 1,2-bis-(2-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid acetoxymethyl ester (BAPTA-AM; Sigma), was loaded onto the Ca^{2+}-free medium. BAPTA-AM (30 mM) was made using a stock solution, with DMSO at a final concentration of 1 mM in a bath solution [11]. BAPTA-AM was loaded onto the neuron for 45 min before lidocaine administration.

We also added a Ca^{2+} ionophore, Calcimycin A23187 (Sigma), to the dish, at a concentration of $5 \mu M$, to identify the relationship between the increased intracellular Ca^{2+} concentration and the neuronal lysis observed as a morphological change resulting from lidocaine [12]. We compared the Ca^{2+} concentrations and morphological pictures before and 60 min after administration of the Ca^{2+} ionophore. Morphological pictures were also taken every 1 h until the cell damage was observed.

Trypan blue 0.4% was added to the dishes, at a final concentration of 0.05%, to clarify the damage to the cell membrane. Morphological pictures were compared before and 1 h after trypan blue addition to each dish to confirm clear staining and cell damage.

Statistical analysis

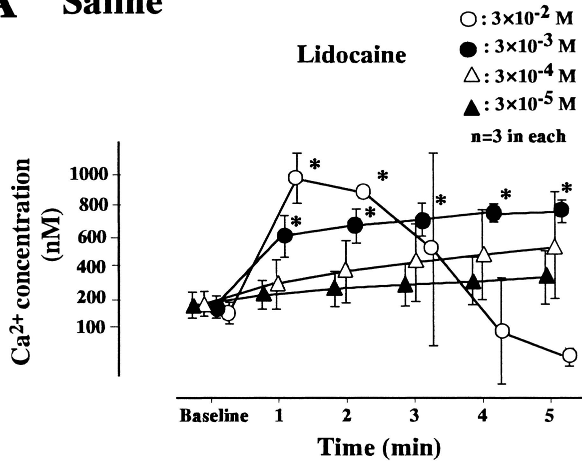
The Ca^{2+} concentrations are presented as means ± SD. Data were analyzed by one-way analysis of variance (ANOVA) for repeated measures with the Bonferroni/Dunn procedure. Comparisons between the two groups at highest concentrations in each experiment, with or without Ca^{2+}, BAPTA-AM, or Ca^{2+} ionophore, were analyzed by Student's *t*-test. All analyses were performed using StatView (Abacus, Berkeley, CA, USA). A value of $P < 0.05$ was considered statistically significant.

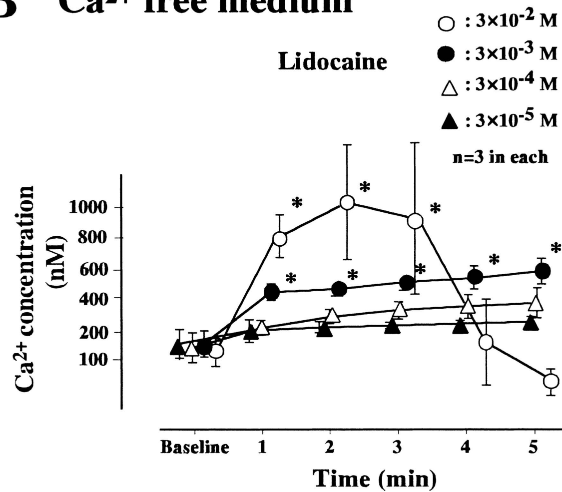
Results

The relationship between the morphological changes in the cells and the lidocaine concentration is shown in Fig. 1.

Lidocaine significantly increased the Ca^{2+} concentration dose-dependently in the normal saline and the Ca^{2+}-free media. The increase in intracellular Ca^{2+} concentration was higher in the saline (from 229 ± 63 to 717 ± 100 nM at 2 min) than in the Ca^{2+}-free medium (from 174 ± 75 to 478 ± 51 nM at 2 min) at 3×10^{-3} M of lidocaine. There were no clear morphological changes, and trypan blue showed no staining of the cells at this Ca^{2+} concentration. When lidocaine was increased to 3×10^{-2} M, the Ca^{2+} concentrations increased significantly, from 238 ± 63 to 1038 ± 156 nM in the saline and from 211 ± 97 to 1046 ± 169 nM in the Ca^{2+} free medium, and the fluorescence of fura-2 then rapidly decreased (Fig. 2A,B). The cell body showed morphological shrinkage and showed the formation of rugged surfaces in both saline and the Ca^{2+}-free medium. These morphologically changed cells were stained dark blue by trypan blue dye. After the use of BAPTA-AM, lidocaine, at 3×10^{-2} M, moderately increased the Ca^{2+} concentration (from 150 ± 97 to 428 ± 246 nM) and produced morphological changes such as shrinkage and the formation of rugged surfaces (Fig. 2C). The Ca^{2+} concentration after the use of BAPTA-AM was significantly lower than those in the other experiments. These morphologically changed cells were also stained by trypan blue.

The Ca^{2+} ionophore gradually increased the Ca^{2+} concentration, from 277 ± 192 to 1323 ± 67 nM (maximum concentration in each cell at 37 ± 16 min) and decreased the Ca^{2+} concentration to 186 ± 109 nM 60 min after Ca^{2+} ionophore administration (Fig. 3A). Morphological examination of the cells revealed only slight swelling at 60 min, but damage became apparent a few hours (3.2 ± 1.9 h) later (Fig. 3B).





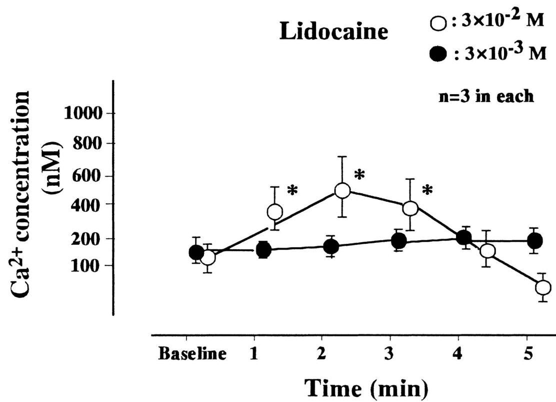


Fig. 2A–C. Intracellular Ca^{2+} concentrations and morphological changes after lidocaine or lidocaine following BAPTA-AM loading. **A** Changes in the intracellular Ca^{2+} concentration after lidocaine administration in normal saline. **B** Changes in the intracellular Ca^{2+} concentration after lidocaine administration in Ca^{2+}-free medium. **C** Changes in the intracellular Ca^{2+} concentration after lidocaine administration following BAPTA-AM loading. * $P < 0.05$ vs baseline

Discussion

Increasing doses of lidocaine increased the intracellular Ca^{2+} concentration and the damage to the cells in our study; however, the intracellular calcium chelator, BAPTA-AM, which prevented the increase in intracellular Ca^{2+} concentration, did not prevent the lidocaine-induced morphological damage. Furthermore, cells with the high Ca^{2+} concentration elevated by the Ca^{2+} ionophore only showed slight swelling for a few hours. These results suggested that morphological damage was not always correlated with the intracellular Ca^{2+} concentration. The increase in intracellular Ca^{2+} concentration is not the only cause of lidocaine-induced cell damage in the cultured neurons of *Lymnaea stagnalis*.

In this experiment, we showed that increasing the dose of lidocaine increased the intracellular Ca^{2+} concentration and produced cell damage in both saline and Ca^{2+}-free media. The lidocaine-induced increase in Ca^{2+} concentration results from Ca^{2+} influx through the plasma membrane and Ca^{2+} release from intracellular stores. Our results showed that increasing the intracellular Ca^{2+} concentration was related to the morphological damage induced by lidocaine. These considerations were generally consistent with those of Gold et al. [3], who suggested that the increase in the Ca^{2+} concentration could be a mechanism of lidocaine-induced neurotoxicity, detected by membrane depolarization using primary cultures of adult rat dorsal root ganglion. Johnson et al. [4] suggested that lidocaine, at levels higher than 2.5%, elevated the intracellular Ca^{2+} concentration to a toxic level that caused a sustained influx of Ca^{2+} through the plasma membrane and released Ca^{2+} from the endoplasmic reticulum.

According to a previous report [13], intracellular Ca^{2+} concentrations following the electrical stimulation of *Lymnaea stagnalis* cells increased to 500 nM in each cell. In the present study, lidocaine at 3×10^{-3} M increased the intracellular Ca^{2+} concentration to levels higher than 700 nM in the saline medium, but showed no morpho-

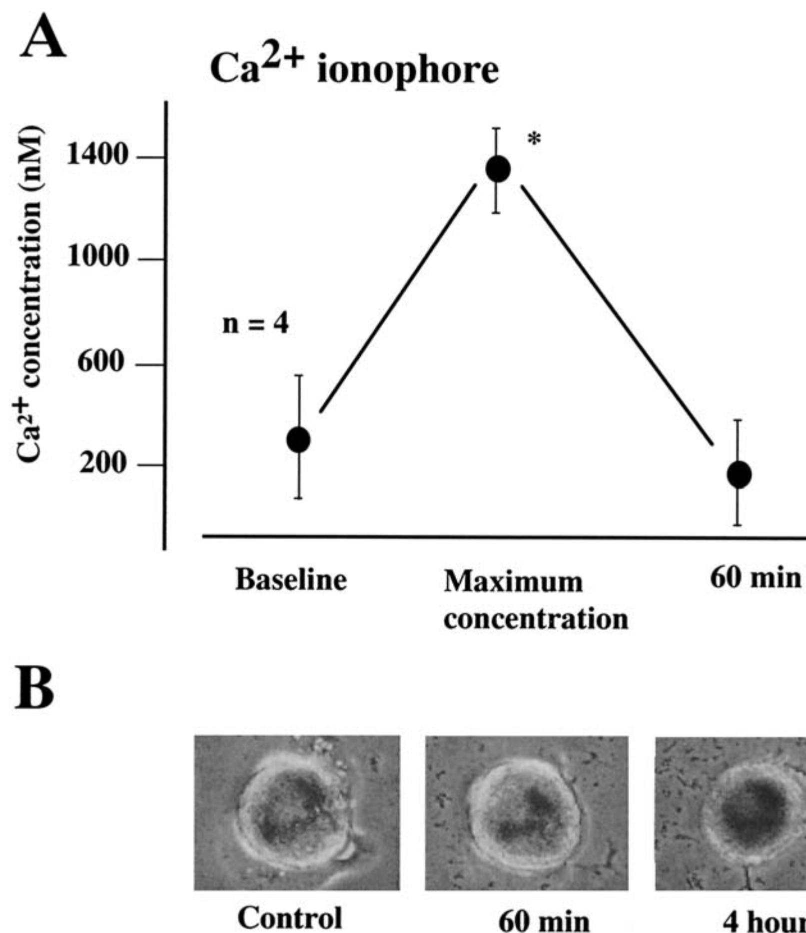


Fig. 3A,B. Intracellular Ca^{2+} concentration and morphological changes after Ca^{2+} ionophore loading. **A** Intracellular Ca^{2+} concentration before (*base line*), maximum concentration during recording, and concentration 60 min after Ca^{2+} ionophore administration. **B** Morphology is pictured before (*control*) and after (*60 min*) recording of the intracellular Ca^{2+} concentration. Morphological changes were observed every hour until cell damage was seen (*4 h* in this cell). $^{*}P < 0.05$ vs baseline

logical damage in the cells. Lidocaine at a concentration of 3×10^{-2} M, resulted in intracellular Ca^{2+} concentrations of more than 1000 nM and a loss of fura-2 fluorescence imaging, which indicated a loss of plasma membrane integrity, in both the saline and the Ca^{2+}-free medium. These morphological changes indicated cell membrane destruction, which was also shown by trypan blue that stained the cell. We also found that the use of a Ca^{2+} ionophore without lidocaine in our experiments resulted in the elevation of the Ca^{2+} concentration to more than 1000 nM, and cytotoxic levels that were revealed after a few hours' observation. However, the cells showed only slight swelling and no morphological damage in the short time period (for the first 60 min) during the lidocaine-loading experiments. Gold et al. [3] reported that BAPTA loading, buffering the lidocaine-induced increase in Ca^{2+}, significantly attenuated lidocaine-induced neuronal death. In our experiments, after loading the Ca^{2+} chelator BAPTA-AM, the Ca^{2+} concentration (<500 nM) was very much lower than the toxic level; however, lidocaine still damaged the cells. Thus, it was indicated that other mechanisms are involved in the neurotoxicity of lidocaine that are not related to the Ca^{2+} concentration. The present results indicate that lidocaine, at concentrations higher than 3×10^{-2} M, has a toxic effect on the cell membrane, with no relation to the Ca^{2+} concentration. Saito et al. [14] showed that tetracaine simultaneously caused a collapse of growth cones and increased the Ca^{2+} concentration in the growth cones. They observed that the growth cones collapsed with no increase in the Ca^{2+} concentration when they loaded BAPTA in Ca^{2+}-free media; they concluded that the collapse of the growth cones and increase of the Ca^{2+} concentration in the cones might have been caused by independent factors. These findings also indicate there are other mechanisms involved in the neurotoxicity of lidocaine that are not related to the Ca^{2+} concentration.

Tan et al. [15] demonstrated that local anesthetics disrupted calcium homeostasis and induced cell death in cultured neuronal cell lines. They reported that an increase in intracellular Ca^{2+} caused the condensation of chromatin, resulting in cell apoptosis. There are two mechanisms, necrosis and apoptosis, that destroy cells; their definition is based on morphological and biological criteria. Apoptosis is characterized by cellular shrinkage, membrane blebbing, nuclear condensation, and internucleosomal DNA fragmentation. Necrosis is

characterized by rapid cell swelling and cell lysis. We found that a high concentration of lidocaine rapidly damaged the cell membrane, resulting in shrinkage and the formation of rugged surfaces. These findings were similar to those of apoptosis, but nuclear condensation was not observed in the cells. In this experiment, we showed that an increasing dose of lidocaine increased the intracellular Ca^{2+} concentration and produced cell damage in both saline and Ca^{2+}-free media. From these results, it seems that the lidocaine-induced increase in Ca^{2+} concentration results from Ca^{2+} influx through the plasma membrane and Ca^{2+} release from intracellular stores. The time course for apoptotic cell death is longer than that seen in our study. In our experiments, we studied the effects of lidocaine for 5 min. Although we were unable to confirm some lidocaine-induced changes, the damage to cells induced by lidocaine observed in this study could primarily be attributed to necrosis. It is generally considered that lidocaine affects the intracellular signaling pathways and cellular plasma membrane of neuronal structures [8]; therefore, it may be natural to consider that apoptosis was also progressing simultaneously.

In our present experiments, we showed that morphological changes were not always correlated with the intracellular Ca^{2+} concentration in the cell body. The interpretation of the current findings has some limitations. Morphological changes in the cultured neurons were assigned scores indicating no change (score 0) to severe change (score 2), the damage mainly occurred with lidocaine, at 3×10^{-2} M, when all cells were damaged rapidly. Comparisons using many cultured cells in a dish and observations for a longer time with increasing doses of lidocaine could be used to analyze cell damage quantitatively. This would reveal in more detail the relationship between morphological change and intracellular Ca^{2+} concentration. More time is required to study whether the mechanisms of lidocaine neurotoxicity have a relationship to the intracellular Ca^{2+} concentration.

Although increasing the intracellular Ca^{2+} concentrations was toxic to the cells, a high concentration of lidocaine showed another mechanism of neurotoxicity. These results indicated that control of the increase in the intracellular Ca^{2+} concentration is insufficient to prevent the neurotoxicity of lidocaine.

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