

- *Short Communication* -

## Effects of tetracaine and lidocaine on intracellular Zn<sup>2+</sup> levels in rat neurons: Preliminary analysis with FluoZin-3 fluorescence

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### Abstract

There are some differences in cytotoxic actions between local anesthetics. In a study performed using rat thymocytes, lidocaine increased intracellular Zn<sup>2+</sup> concentration whereas tetracaine decreased it. However, thymocytes are not subject to local anesthetics. This study was conducted to confirm the actions of tetracaine and lidocaine on neurons. Tetracaine and lidocaine changed intracellular Zn<sup>2+</sup> levels in rat cerebellar granule neurons, as had been observed in rat thymocytes. The changes in intracellular Zn<sup>2+</sup> levels by these local anesthetics were statistically significant, but small. The mechanism of lidocaine-induced increase in intracellular Zn<sup>2+</sup> levels in neurons was similar to that in rat thymocytes. The mechanism of tetracaine-induced decrease in intracellular Zn<sup>2+</sup> levels in neurons did not seem to be consistent with that in rat thymocytes. Further studies will be needed to see if the changes in intracellular Zn<sup>2+</sup> levels by local anesthetics correspond to differences in cytotoxic activities since their actions on intracellular Zn<sup>2+</sup> levels of neurons were weak.

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### 1. Introduction

In the study using rat thymocytes, tetracaine decreased intracellular Zn<sup>2+</sup> levels by attenuating temperature-sensitive Zn<sup>2+</sup> influx (Kimura et al., 2011). In contrast, lidocaine increases intracellular Zn<sup>2+</sup> levels (Nishimura et al., 2010, 2012). These results may reveal the difference in the actions between local anesthetics even though lymphocytes are not subject to local anesthetics.

Zinc affects enzymatic activity and several signaling molecules (Vallee and Auld, 1993; Prasad, 1995). Furthermore, zinc acts as an intracellular second messenger (Yamasaki et al., 2007). There are many studies supporting the physiological and nutritional roles of zinc at present. However, zinc at micromolar concentrations augments the cytotoxic activities of triazole antifungals (Matsui et al., 2008; Kinazaki et al., 2009), polysorbate 80 (Oyama et al., 2010), and hydrogen peroxide (Matsui et al., 2010) on rat thymocytes under in vitro conditions. In addition, some cytotoxic chemicals significantly increase intracellular Zn<sup>2+</sup> levels (Oyama et al., 2009; Kawanai

et al., 2009; Tamura et al., 2012). Thus, zinc may also be involved in the cytotoxic effects of some chemical substances.

Local anesthetics exert cytotoxic effects on neuronal cells; these effects are concentration and exposure time dependent (Kanai et al., 2000; Johnson et al., 2002). Cytotoxic effects of some local anesthetics are presumed to be related to an increase in intracellular Ca<sup>2+</sup> levels (Gold et al., 1998; Kasaba, 2007). However, although zinc possesses various physiological roles, there is little information concerning the effects of local anesthetics on intracellular Zn<sup>2+</sup> levels in neuronal cells. There are some differences between the cytotoxic effects of local anesthetics (Johnson et al., 2002; Perez-Castro et al., 2009; Lee et al., 2009). In the study using lymphocytes, lidocaine increased cytosolic Zn<sup>2+</sup> levels whereas tetracaine decreased them (Nishimura et al., 2010, 2012; Kimura et al., 2011). If this were the case in neuronal cells, the results would promote a better understanding on the difference in cytotoxic actions (or neurotoxicity) between local anesthetics. In this study, we examined the effects of tetracaine

and lidocaine on intracellular  $Zn^{2+}$  levels in rat cerebellar granule neurons.

## 2. Methods

### 2.1. Chemicals

Tetracaine and lidocaine were purchased from Sigma Chemicals (St. Louis, MO, USA). FluoZin-3-AM, 5-chloromethylfluorescein diacetate (5-CMF-DA), and propidium iodide were purchased from Molecular Probes, Inc. (Eugene, OR, USA). The chelators, diethylenetriamine- $N,N,N',N'',N''$ -pentaacetic acid (DTPA) for extracellular  $Zn^{2+}$  and  $N,N,N',N'$ -tetrakis[2-pyridylmethyl]ethylenediamine (TPEN) for intracellular  $Zn^{2+}$ , were obtained from Dojin Chemical Laboratory (Kumamoto, Japan). The pH buffer was 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Nacalai Tesque, Kyoto, Japan). Other chemicals were purchased from Wako Pure Chemicals.

### 2.2. Dissociation of rat cerebellar granule neurons

Rat cerebellum was sliced at a thickness of 500  $\mu$ m. The slices were treated with dispase for 60 min. After enzymatic treatment, they were triturated in Tyrode's solution to disperse the neurons. Tyrode's solution containing dissociated cerebellar neurons was passed through a filter to remove large neurons and tissue fragments.

Strictly speaking, cerebellar granule neurons are not subject to local anesthetics. However, some local anesthetics induce transitory cerebellar ataxia and convulsion. Therefore, some side effects of local anesthetics may be related to their actions on cerebellar neurons. In addition, for technical considerations, the experiments involving flow cytometry require a large number of cells. Enzymatic treatment of cerebellar tissue provides a large number of intact granule neurons (i.e., a large volume of cell suspension).

### 2.3. Measurement of FluoZin-3 fluorescence (presumably intracellular $Zn^{2+}$ level)

FluoZin-3-AM was added to the cell suspension to achieve a final concentration of 500 nM. Neurons were incubated with FluoZin-3-AM for at least 60 min before measurement of FluoZin-3 fluorescence, an indicator of intracellular  $Zn^{2+}$  (Gee et al., 2002). Measurements were performed with a flow cytometer

(CytoACE, JASCO, Tokyo, Japan). FluoZin-3 fluorescence was measured in cells that were not stained with 5  $\mu$ M propidium iodide. The excitation wavelength for FluoZin-3 was 488 nm, and emission was detected at a wavelength of  $530 \pm 20$  nm.

### 2.4. Measurement of 5-chloromethylfluorescein fluorescence (presumably cellular content of nonprotein thiols)

5-CMF-DA was used to monitor changes in the cellular content of nonprotein thiols, presumably glutathione (Chikahisa et al., 1996). The cells were incubated with 500 nM 5-CMF-DA for 30 min before any fluorescence measurements. 5-CMF fluorescence was measured in cells that were not stained with 5  $\mu$ M propidium iodide. The excitation wavelength used for 5-CMF was 488 nm and emission was detected at  $530 \pm 15$  nm.

### 2.5. Protocol

Local anesthetics (lidocaine and tetracaine) were added to the cell suspension. The cell density was approximately  $5 \times 10^5$  cells/mL. The cells were incubated with local anesthetics for appropriate times under several experimental conditions.

### 2.6. Statistics

Values were expressed as the mean  $\pm$  standard deviation of 4 samples. Statistical analysis was performed with Tukey's multivariate analysis. A P value of  $<0.05$  was considered significant.

## 3. Results and Discussion

### 3.1. Effects of local anesthetics on the intensity of FluoZin-3 fluorescence

As shown in Fig. 1, the incubation of cells with 0.1–1 mM tetracaine for 1 h decreased the intensity of FluoZin-3 fluorescence while the reverse was the case for 3 mM lidocaine. These changes were small, but statistically significant. The result suggests that tetracaine decreases intracellular  $Zn^{2+}$  concentration in neurons while lidocaine decreases it. Thus, tetracaine exerted an action that was opposite to that of lidocaine, as described in rat thymocytes (Kimura et al., 2011).

## Effects of tetracaine and lidocaine on intracellular Zn<sup>2+</sup> levels

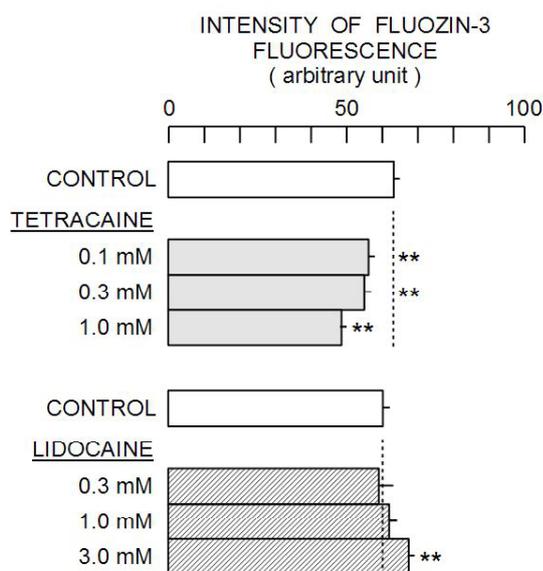


Figure 1. Changes in the intensity of FluoZin-3 fluorescence by tetracaine and lidocaine. Each column and bar respectively indicates mean and standard deviation of four samples. Dotted line represents the control level. Symbol \*\* indicates a significant difference between the control group and test group.

### 3.2. Local anesthetic action in the presence of Zn<sup>2+</sup> chelators

To determine whether external Zn<sup>2+</sup> is involved in the actions of tetracaine and lidocaine, the effects of 1 mM tetracaine and 3 mM lidocaine (1 h incubation) on the intensity of FluoZin-3 fluorescence in the presence of 3 μM DTPA, a chelator of extracellular Zn<sup>2+</sup>. In the presence of DTPA, the actions of tetracaine and lidocaine were not changed, although DTPA decreased the control level of FluoZin-3 fluorescence (Fig. 2). It is unlikely that external Zn<sup>2+</sup> is involved in the actions of tetracaine and lidocaine.

TPEN, a chelator of intracellular Zn<sup>2+</sup>, greatly decreased the intensity of FluoZin-3 fluorescence. Tetracaine slightly reduced the intensity of FluoZin-3 fluorescence while lidocaine did not affect FluoZin-3 fluorescence in the presence of TPEN. The changes in FluoZin-3 fluorescence by tetracaine and lidocaine are presumably dependent on the changes in intracellular Zn<sup>2+</sup> levels.

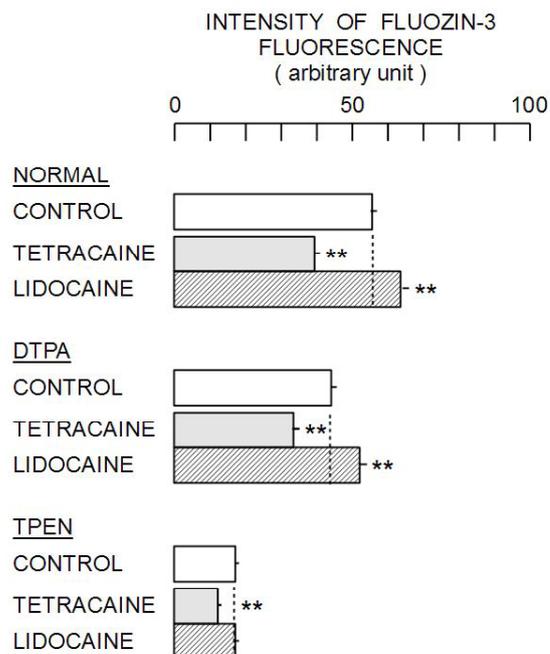


Figure 2. Effects of 1 mM tetracaine and 3 mM lidocaine on the intensity of FluoZin-3 fluorescence in the presence of a Zn<sup>2+</sup> chelator. Each column and bar respectively indicates mean and standard deviation of four samples. Dotted line represents the control level. Symbol \*\* indicates a significant difference between the control group and test group.

### 3.3. Effect on the intensity of 5-CMF fluorescence

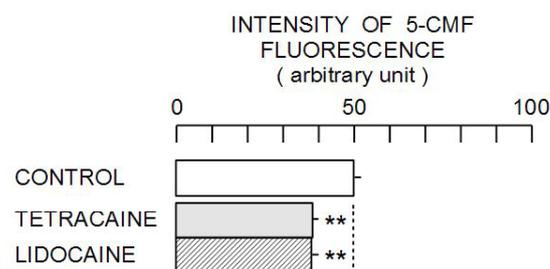


Figure 3. Effects of 1 mM tetracaine and 3 mM lidocaine on the intensity of 5-CMF fluorescence. Each column and bar respectively indicates mean and standard deviation of four samples. Dotted line represents the control level. Symbol \*\* indicates a significant difference between the control group and test group.

To determine whether tetracaine and lidocaine affect cellular content of nonprotein thiols, the effects of local anesthetics on 5-CMF fluorescence, a parameter of nonprotein thiol levels (Chikahisa et al., 1996) were examined. Both 1 mM tetracaine and 3 mM lidocaine equally decreased the intensity of 5-CMF fluorescence after 1 h incubation with the respective agents (Fig. 3).

Recent studies reveal that chemical compounds possessing pro-oxidant activity increase intracellular  $Zn^{2+}$  levels with a corresponding decrease in the cellular content of nonprotein thiols (Kawanai et al., 2009; Kinazaki et al., 2011; Tamura et al., 2012). The conversion from thiols to disulfides by oxidative stress releases  $Zn^{2+}$ , resulting in an increase in intracellular  $Zn^{2+}$  levels (Maret, 1994). Lidocaine-induced increases in intracellular  $Zn^{2+}$  levels may be explained by  $Zn^{2+}$  release from thiols. However, tetracaine decreased both FluoZin-3 and 5-CMF fluorescence (Figs. 1 and 3). The decrease

in cellular content of nonprotein thiols is presumed to induce the increase in intracellular  $Zn^{2+}$  levels as described above. This is not consistent with the case of 1 mM tetracaine in neurons. In rat thymocytes, tetracaine decreased temperature-sensitive  $Zn^{2+}$  influx, possibly resulting in a decrease in intracellular  $Zn^{2+}$  levels (Kimura et al., 2011). However, DTPA, a chelator of extracellular  $Zn^{2+}$ , did not affect the tetracaine-induced decrease in the intensity of FluoZin-3 fluorescence (Fig. 2). Therefore, the change in intracellular  $Zn^{2+}$  levels by tetracaine cannot be explained by the mechanism proposed in rat thymocytes. The mechanism will be elucidated in future studies.

The changes in intracellular  $Zn^{2+}$  levels by tetracaine and lidocaine were small (Fig. 1). Therefore, it is quite difficult to argue that the difference in cytotoxic activities of local anesthetics is due to their different actions on intracellular  $Zn^{2+}$  levels.

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