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Further analysis on lidocaine-induced increase in intracellular Zn^{2+} concentration: Cytometric model study using FluoZin-3, 5-chloromethylfluorescein, and rat thymocytes

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Abstract

Lidocaine augmented FluoZin-3 fluorescence of rat thymocytes, suggesting the increase in intracellular Zn^{2+} concentration. However, the mechanism for lidocaine-induced increase in the intensity of FluoZin-3 fluorescence was not elucidated. In this study, in order to reveal the mechanism, the effects of lidocaine on FluoZin-3 and 5-chloromethylfluorescein (5-CMF) fluorescence were examined under various conditions. 5-CMF fluorescence was used as a parameter for cellular content of nonprotein thiols. The lidocaine-induced augmentation of FluoZin-3 fluorescence was observed under external Ca^{2+}- and/or Zn^{2+}-free conditions, suggesting that lidocaine induced intracellular Zn^{2+} release. Lidocaine attenuated 5-CMF fluorescence, suggesting the decrease in cellular content of nonprotein thiols. Since some peptides consisting of SH-group have been proposed to store Zn^{2+}, the decrease in nonprotein thiol content by lidocaine reduced the cellular ability to store Zn^{2+}, leading to an increase in intracellular Zn^{2+} concentration.

Key words: Lidocaine; Intracellular Zn^{2+} level; FluoZin-3

1. Introduction

Both lidocaine and tetracaine are well known as representative local anesthetics. In our previous studies, lidocaine augmented FluoZin-3 fluorescence of rat thymocytes (Nishimura and Oyama, 2009) whereas tetracaine attenuated it (Kimura et al., 2011). These results suggested that lidocaine increased intracellular Zn^{2+} concentration while tetracaine decreased it. Tetracaine was supposed to attenuate an influx of external Zn^{2+}, resulting in the decrease in intracellular Zn^{2+} concentration (Kimura et al., 2011). However, the mechanism for the lidocaine-induced augmentation of FluoZin-3 fluorescence (presumably, the lidocaine-induced increase in intracellular Zn^{2+} concentration) was not elucidated.

In this study, the effect of lidocaine on FluoZin-3 fluorescence was examined under Ca^{2+}- and/or Zn^{2+}-free conditions to reveal the contribution of intracellular Zn^{2+} release to the lidocaine-induced augmentation of FluoZin-3 fluorescence. Furthermore, the lidocaine-induced change in 5-chloromethylfluorescein (5-CMF) fluorescence, a parameter for cellular content of nonprotein thiols, was examined to clarify the source of released Zn^{2+} because there was negative correlation between the increase in intracellular Zn2+ concentration and the decrease in cellular thiol content during oxidative stress (Matsui et al., 2010)

2. Materials and methods

2.1. Chemicals

NaCl, $CaCl_2$, $MgCl_2$, KCl, glucose, HEPES, NaOH, and dimethyl sulfoxide (DMSO) were obtained from Wako Pure Chemicals (Osaka, Japan). FluoZin-3-AM, propidium iodide, and 5-CMF diacetate (5-CMF-DA) were obtained from Molecular Probes Inc. - Invitrogen (Eugene, Oregon, USA). Propidium iodide was dissolved with 50 % ethanol. FluoZin-3-AM and 5-CMF-DA were dissolved in DMSO. The final concentrations of solvents (0.1 % DMSO and 0.05% ethanol) in the cell suspension did not affect the cell viability and any fluorescence. Diethylenetriamine-*N,N,N′,N″,N″*-pentaacetic acid (DTPA), a chelator for external Zn^{2+}, was obtained from Dojin Chemical Laboratory (Kumamoto, Japan).

2.2. Animals and cell preparation

This study was approved by the Committee for Animal Experiments at the University of Tokushima (No. 05279). The cell suspension was prepared in a similar manner to that reported previously (Chikahisa et al., 1996; Matsui et al., 2008; Kinazaki et al., 2011). In brief, thymus glands dissected from

ether-anesthetized rats were sliced at a thickness of 1–2 mm with a blade under cold conditions (3–4°C). The slices were triturated by gently shaking in Tyrode's solution to dissociate the thymocytes. Thereafter, the Tyrode's solution containing the cells was passed through a mesh (diameter 10 μm) to prepare the cell suspension. The beaker containing the cell suspension was incubated in a water bath at 36–37°C for 1 h before the experiment. Although the Tyrode's solution did not contain $ZnCl_2$, the cell suspension generally contained 200–230 nM zinc derived from the cell preparation (Sakanashi et al., 2009).

2.3. Fluorescence measurements of cellular and membrane parameters

The methods for measuring the cellular and membrane parameters by using a flow cytometer equipped with an argon laser (CytoACE-150; JASCO, Tokyo, Japan) and fluorescent probes were similar to those described previously (Chikahisa et al., 1996; Matsui et al., 2008). The fluorescence was analyzed by JASCO software (Version 3.06; JASCO). To assess cell lethality, propidium iodide was added to the cell suspensions to a final concentration of 5 μM. Because propidium stains dead cells, the measurement of propidium fluorescence from the cells provided information on lethality. The fluorescence was measured 2 min after the application of propidium iodide by a flow cytometer. The excitation wavelength used for propidium was 488 nm and the emission was detected at 600 ± 20 nm. FluoZin-3-AM (Gee et al., 2002) was used as an indicator of intracellular Zn^{2+}. The cells were incubated with 500 nM FluoZin-3-AM for 60 min before any fluorescence measurements were taken to estimate the change in the intracellular Zn^{2+} level of the rat thymocytes with intact membranes. FluoZin-3 fluorescence was measured in the cells that were not stained with propidium iodide (Matsui et al., 2008). The excitation wavelength used for FluoZin-3 was 488 nm and the emission was detected at 530 ± 20 nm. 5-CMF-DA was used to monitor changes in the cellular content of nonprotein thiols (Chikahisa et al., 1996). The cells were incubated with 1 μM 5-CMF-DA for 30 min before any fluorescence measurements were taken. 5-CMF fluorescence was measured in the cells that were not stained with propidium iodide. The excitation wavelength used for 5-CMF was 488 nm, and the emission was detected at 530 ± 15 nm.

2.4. Statistics

Values were expressed as the mean ± standard deviation of 4 experiments. Statistical analysis was performed with Scheffe's multivariate analysis. A P value of <0.05 was considered significant.

3. Results

3.1. The change in FluoZin-3 fluorescence of rat thymocytes by lidocaine

The incubation with 0.3 mM lidocaine for 1 h did not affect the FluoZin-3 fluorescence. Lidocaine at 1 mM started to increase the intensity of FluoZin-3 fluorescence. The increase by 1 mM lidocaine was slight, but statistically-significant. When the cells were incubated with 3 mM or 10 mM lidocaine for 1 hr, further augmentation of FluoZin-3 fluorescence was observed. The results were summarized in Fig. 1. Thus, the incubation with lidocaine at concentrations ranging from 1 mM to 10 mM concentration-dependently increased the intensity of FluoZin-3 fluorescence.

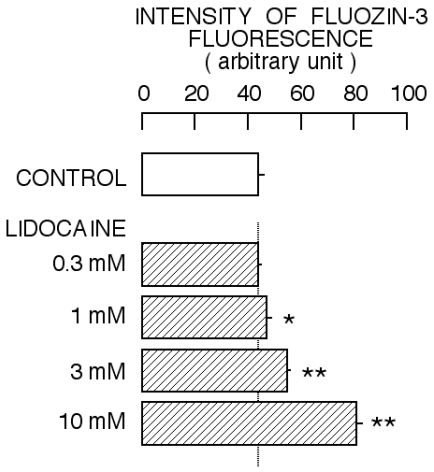

Figure 1. Concentration-dependent change in FluoZin-3 fluorescence by lidocaine. Effect was examined 60 min after the start of application of lidocaine. Columns and bars indicate the means and standard deviations of 4 experiments, respectively. The symbols (, **) indicate significant differences (P < 0.05, P < 0.01) between the control group and the lidocaine-treated groups.*

3.3. The change in FluoZin-3 fluorescence by lidocaine under external Ca^{2+}-free and Zn^{2+}-free conditions

In previous study (Nishimura and Oyama, 2010), lidocaine at 10 mM significantly increased intracellular Ca^{2+} concentration and this increase by lidocaine was greatly dependent on extracellular Ca^{2+}. To remove the contribution of the lidocaine-induced increase in intracellular Ca^{2+} concentration to the lidocaine-induced augmentation of FluoZin-3 fluorescence, the effect of lidocaine was examined under nominal Ca^{2+}-free condition.

Removal of $CaCl_2$ from external solution significantly increased the intensity of FluoZin-3 fluorescence and lidocaine at 10 mM further augmented the FluoZin-3 fluorescence (Fig. 2). The

augmentative action of lidocaine on the FluoZin-3 fluorescence was similarly observed under nominal Ca^{2+}-free condition. The incubation solution contained small amount (200–230 nM) of Zn^{2+} that was delivered from the cell preparation (Sakanashi et al., 2009). The experiment was conducted to examine the contribution of external Zn^{2+} to the lidocaine-induced augmentation of FluoZin-3 fluorescence. The application of 3 μM DTPA, a chelator for extracellular Zn^{2+}, decreased the intensity of FluoZin-3 fluorescence under nominal Ca^{2+}-free condition (Fig. 2). Further application of 10 mM lidocaine significantly increased the intensity of FluoZin-3 fluorescence in the presence of DTPA. Thus, the augmentation of FluoZin-3 fluorescence by lidocaine was observed under Ca^{2+}- and Zn^{2+}-free condition.

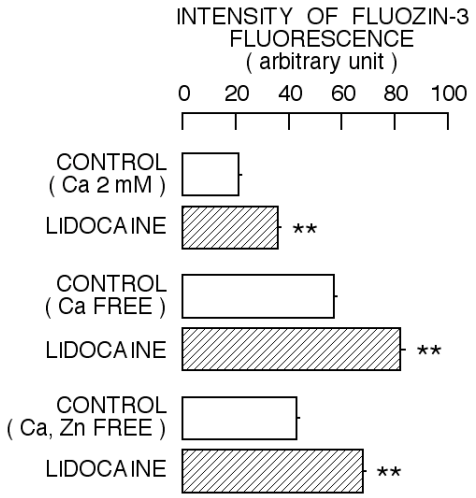

Figure 2. Effect of lidocaine on FluoZin-3 fluorescence under Ca^{2+}-free and/or Zn^{2+}-free conditions. Effect was examined 60 min after the start of application of lidocaine. Columns and bars indicate the means and standard deviations of 4–8 experiments, respectively. The symbol (**) indicates significant difference ($P < 0.01$) between the control group and the lidocaine-treated group under respective condition.

3.3. The change in 5-CMF fluorescence of rat thymocytes by lidocaine

From the results of Fig. 2, lidocaine was supposed to increase intracellular Zn^{2+} concentration, independent from external Zn^{2+}. Some agents that decreased cellular content of nonprotein thiols were supposed to increase intracellular Zn^{2+} concentration (Hashimoto et al., 2009; Oyama et al., 2009; Kinazaki et al., 2011; Tamura et al., 2011). It was reminiscent of the possibility that lidocaine induced the release of Zn^{2+} from nonprotein zinc-binding thiols. To test the possibility, the change in 5-CMF fluorescence by lidocaine was examined. As shown in Fig. 3, the incubation with 10 mM lidocaine significantly decreased the intensity of 5-CMF fluorescence. Thus, lidocaine augmented the FluoZin-3 fluorescence whereas it attenuated the 5-CMF fluorescence.

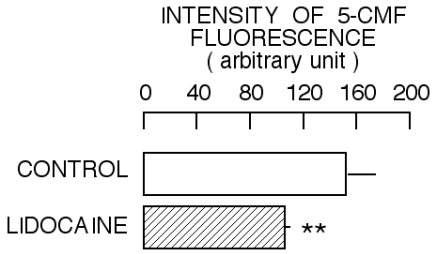

Figure 3. Effect of lidocaine on 5-CMF fluorescence. The cells were treated with lidocaine for 60 min and thereafter 5-CMF-DA was applied to the cell suspension. Effect was examined 30 min after the start of application of 5-CMF-DA. Therefore, the time for lidocaine treatment was 90 min. Columns and bars indicate the means and standard deviations of 4 experiments, respectively. The symbol (**) indicates significant difference ($P < 0.01$) between the control group and the lidocaine-treated group.

4. Discussion

Milimolar concentrations (1–10 mM) of lidocaine significantly increased the intensity of FluoZin-3 in a concentration-dependent manner (Fig. 1). The result indicated the lidocaine-induced increase in intracellular Zn^{2+} concentration (Nishimura and Oyama, 2010; Kimura et al., 2011). Removal of external Ca^{2+} increased the intensity of FluoZin-3 fluorescence under control condition (Fig. 2). The mechanism for the augmentation of FluoZin-3 fluorescence by external Ca^{2+} removal was not elucidated in this study. Lidocaine further increased the intensity of FluoZin-3 fluorescence under nominal Ca^{2+}-free condition (Fig. 2). Thus, external Ca^{2+} was not involved in the lidocaine-induced augmentation of FluoZin-3 fluorescence. It was noted that the cell suspension contained 200–230 nM Zn^{2+} that was delivered from cell preparation although there was no $ZnCl_2$ in the composition of Tyrode's solution (Sakanashi et al., 2009). The application of DTPA, a chelator for external Zn^{2+}, decreased the intensity of FluoZin-3 fluorescence under nominal Ca^{2+}-free condition (Fig. 2). Thus, the Zn^{2+} influx was supposed to maintain the level of intracellular Zn^{2+}. Lidocaine also increased the intensity of FluoZin-3 fluorescence under external Ca^{2+}- and Zn^{2+}-free condition (Fig. 2), indicating that external Zn^{2+} was not involved in the lidocaine-induced increase in the intensity of FluoZin-3 fluorescence. Therefore, it was suggested

that lidocaine increased intracellular Zn^{2+} concentration that was independent from external Zn^{2+}.

Oxidative stress by hydrogen peroxide increased the intensity of FluoZin-3 fluorescence of rat thymocytes (Matsui et al., 2010). The application of hydrogen peroxide greatly attenuated the 5-CMF fluorescence (Chikahisa et al., 1996). There was also reverse correlation between the attenuation of 5-CMF fluorescence and the augmentation of FluoZin-3 fluorescence in the cases of thimerosal, tri-*n*-butyltin, N-ethylmaleimide, and triclosan (Hashimoto et al., 2009; Oyama et al., 2009; Kinazaki et al., 2011; Tamura et al., 2011). In this study, lidocaine decreased the intensity of 5-CMF fluorescence whereas it increased that of FluoZin-3 fluorescence. It is presumably suggested that lidocaine decreases the cellular content of nonprotein thiols, resulting in the release of Zn^{2+} that increases intracellular Zn^{2+} concentration.

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References

Chikahisa, L., Oyama, Y., Okazaki, E., Noda, K., 1996. Fluorescent estimation of H_2O_2-induced changes in cell viability and cellular nonprotein thiol level of dissociated rat thymocytes. Jpn. J. Pharmacol. 71, 299–305.

Gee, K.R., Zhou, Z.L., Qian, W.J., Kennedy, R., 2002. Detection and imaging of zinc secretion from pancreatic beta-cells using a new fluorescent zinc indicator. J. Am. Chem. Soc. 124, 776–778.

Hashimoto, E., Oyama, T.B., Oyama, K., Nishimura, Y., Oyama, T.M., Ueha-Ishibashi, T., Okano, Y., Oyama, Y., 2009. Increase in intracellular Zn^{2+} concentration by thimerosal in rat thymocytes: Intracellular Zn^{2+} release induced by oxidative stress. Toxicol. In Vitro. 23, 1092–1099.

Kimura, K., Nishimura, Y., Oyama, K., Kawanai, T., Hashimoto, E., Oyama, Y., 2011. Tetracaine decreases intracellular Zn^{2+} concentration by inhibiting Zn^{2+} influx in rat thymocytes. Nat. Sci. Res. Univ. Tokushima 25, 7–13.

Kinazaki, A., Chen, H., Koizumi, K., Kawanai, T., Oyama, T.M., Satoh, M., Ishida, S., Okano, Y., Oyama, Y., 2011. Putative role of intracellular Zn^{2+} release during oxidative stress: A trigger to restore cellular thiol content that is decreased by oxidative stress. J. Physiol. Sci. 61, 403–409.

Matsui, H., Sakanashi, Y., Oyama, T.M., Oyama, Y., Yokota, S., Ishida, S., Okano, Y., Oyama, T.B., Nishimura, Y., 2008. Imidazole antifungals, but not triazole antifungals, increase membrane Zn^{2+} permeability in rat thymocytes: Possible contribution to their cytotoxicity. Toxicology. 248, 142–150.

Matsui, H., Oyama, T.M., Okano, Y., Hashimoto, E., Kawanai, T., Oyama, Y., 2010. Low micromolar zinc exerts cytotoxic action under H_2O_2-induced oxidative stress: Excessive increase in intracellular Zn^{2+} concentration. Toxicology. 276, 27–32.

Nishimura, Y., Oyama, Y., 2009. Cytotoxic actions of lidocaine at sublethal concentrations: A model in vitro experiment using rat thymocytes. Nat. Sci. Res. Univ. Tokushima. 24, 1–5.

Oyama, T.B., Oyama, K., Kawanai, T., Oyama, T.M., Hashimoto, E., Satoh, M., Oyama, Y., 2009. Tri-*n*-butyltin increases intracellular Zn^{2+} concentration by decreasing cellular thiol content in rat thymocytes. Toxicology. 262, 245–249.

Sakanashi, Y., Oyama, T.M., Matsuo, Y., Oyama, T.B., Nishimura, Y., Ishida, S., Imai, S., Okano, Y., Oyama, Y., 2009. Zn^{2+}, derived from cell preparation, partly attenuates Ca^{2+}-dependent cell death induced by A23187, calcium ionophore, in rat thymocytes. Toxicol. In Vitro. 23, 338–345.

Tamura, I., Kanbara, Y., Saito, M., Horimono, K., Satoh, M., Yamamoto, H., Oyama, Y., 2011. Triclosan, an antibacterial agent, increases intracellular Zn^{2+} concentration in rat thymocytes: Its relation to oxidative stress. Chemosphere. 86, 70–75.

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