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Thiopental elevates steady-state levels of intracellular Ca^{2+} and Zn^{2+} in rat thymic lymphocytes

Keisuke Oyama[1], Shinya Ueno[2], Mizuki Mizobuchi[3], Naoki Kanematsu[3], Kazumi Ishidoh[3], Norio Kamemura[3,*]

[1]Surgery Division, Sakai City Medical Center, Sakai 593-8304, Japan
[2]Department of Neurophysiology, Hirosaki University Graduate School of Medicine, Aomori 036-8562, Japan
[3]Department of Food-Nutritional Sciences, Faculty of Life Sciences, Tokushima Bunri University, Tokushima 770-8514, Japan
*E-mail: kamemura.norio@tks.bunri-u.ac.jp

Abstract

Thiopental is an ultra-short-acting barbiturate and has been used commonly in the induction phase of general anesthesia. However, the toxic effect of thiopental is not completely clear. The effect of thiopental on intracellular Ca^{2+} ($[Ca^{2+}]i$) levels was investigated in non-excitable cells. Experiments were carried out using a flow-cytometric technique, rat thymic lymphocytes (as non-excitable cells), and appropriate fluorescent probes. Treatment of cells with 300 µM thiopental increased Fluo-3 fluorescence intensity, indicating elevation of $[Ca^{2+}]i$. This increase was partially attenuated by a chelator of intracellular Zn^{2+}. Thus, thiopental elevated both $[Ca^{2+}]i$ and intracellular Zn^{2+} ($[Zn^{2+}]i$) levels. Under intracellular Zn^{2+}-free conditions, 100–300 µM thiopental was still able to induce a statistically significant increase in $[Ca^{2+}]i$, whereas removal of extracellular Ca^{2+} greatly reduced the increase in $[Ca^{2+}]i$ induced by this dose of thiopental. Therefore, the thiopental-induced increase in $[Ca^{2+}]i$ was mainly due to an increased influx of Ca^{2+}. Treatment of cells with 300 µM thiopental increased FluoZin-3 fluorescence intensity, indicating the presence of $[Zn^{2+}]i$, both in the presence and absence of extracellular Zn^{2+}. The thiopental-induced elevation of $[Zn^{2+}]i$ was due to an increase in both influx of Zn^{2+} and intracellular Zn^{2+} release. Concanavalin A (10 µg/mL) augmented Fluo-3 fluorescence in the presence of an intracellular Zn^{2+} chelator. The combination of concanavalin A and 100–300 µM thiopental synergistically increased $[Ca^{2+}]i$. Results suggest that thiopental increases $[Ca^{2+}]i$ in both quiescent and activated lymphocytes, possibly resulting in modulation of immune system function.

Keywords: thiopental, intracellular Ca^{2+}, intracellular Zn^{2+}

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■ Introduction

Thiopental is a short-acting barbiturate possessing sedative, hypnotic, and anticonvulsant properties. It is used for induction of anesthesia. The action of thiopental on intracellular Ca^{2+} levels ($[Ca^{2+}]i$) has been mainly investigated in electrically excitable cells such as cardiac myocytes and neurons [1]. Thiopental suppresses voltage-gated Ca^{2+} channels [2-4], resulting in inhibition of the transient increase in $[Ca^{2+}]i$ during propagation of action potentials. Such an inhibition induces alterations in muscle contraction and neurotransmitter release [5, 6]. Ca^{2+} also plays a central physiological role as an intracellular signaling secondary messenger in non-electrically-excitable cells such as lymphocytes [7, 8]. Furthermore, Zn^{2+}, too , is reported to take part in lymphocyte intracellular signaling [9, 10]. In addition, excessive increases in $[Ca^{2+}]i$ and/or $[Zn^{2+}]i$ induce cell death, including via apoptosis [11-14]. There are contradictory papers on the action of thiopental on lymphocyte apoptosis. Thiopental was reported to induce apoptosis [15] and to protect human T lymphocytes from apoptosis in vitro via the expression of heat shock protein 70 [16]. Therefore, a better understanding of the actions of thiopental on intracellular Ca^{2+} and Zn^{2+} levels is necessary for improved drug safety. This study, by making use of a flow-cytometric technique, rat thymic lymphocytes, and fluorescent probes for intracellular Ca^{2+} and Zn^{2+}, demonstrated the actions of thiopental in both quiescent and activated lymphocytes.

■ Experimental

Cell suspension

The use of experimental animals in this study was approved by the committee of Tokushima University and Tokushima Bunri University (Tokushima, Japan) (Registration number in Tokushima university : T29-52, Registration number in Tokushima Bunri university: H30-3). Thymi were dissected from male Wistar rats (6–8 weeks old) which were intraperitoneally anesthetized with thiopental sodium (50 mg/kg). Thymi were immersed in ice-cold normal or Ca^{2+}-free Tyrode's solution prior to razor sectioning. Normal Tyrode's solution was prepared with NaCl (150 mM), KCl (5 mM), $CaCl_2$ (2 mM), $MgCl_2$ (1 mM), 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (5 mM) and the appropriate amount of 2–3 mM NaOH to achieve a pH of 7.3–7.4. Ca^{2+}-free Tyrode's solution was prepared by substituting $CaCl_2$ with equimolar $MgCl_2$. Sectioned thymi were dispersed in Tyrode's solution to dissociate thymic lymphocytes (thymocytes) under cold conditions, in order to obtain a cell suspension. Cells were centrifuged and washed with Tyrode's solution to avoid possible contamination by residual thiopental in the cell suspension. Cells were resuspended such that cell density was appropriate to achieve measurement of 200–300 cells per second during flow-cytometric analysis.

Reagents

Sodium salt of thiopental for clinical use was purchased from Mitsubishi Tanabe Pharma (Osaka, Japan). Thiopental was freshly dissolved in distilled water to a stock concentration of 30–300 mM just prior to use. Thiopental stock solution was added to the cell suspension to achieve final bath concentrations of 30–300 µM. Fluorescent probes used in this study included propidium iodide (Invitrogen, Eugene, Oregon, USA), Fluo-3-AM (Dojin Chemical, Kumamoto, Japan), and FluoZin-3-AM (Invitrogen). Propidium iodide was dissolved in a 50% ethanol solution to a stock concentration of 10 mM. Fluo-3-AM and FluoZin-3-AM, respectively, were dissolved in dimethyl sulfoxide (DMSO; Wako Pure Chemical, Osaka, Japan) to stock concentrations of 2 mM. Final probe concentrations in the cell suspension were two thousand times lower than stock concentrations. Chelators for external Ca^{2+}, external Zn^{2+}, and intracellular Zn^{2+} were, respectively, sodium salt of ethylenediaminetetraacetic acid (EDTA), diethylenetriamine-*N*,*N*,*N'*,*N''*,*N''*-pentaacetic acid (DTPA), and *N*,*N*,*N'*,*N'*-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN). These chelators were obtained from Dojin Chemical. All reagents were products of Wako Chemical unless otherwise specified.

Cytometric measurements

To photochemically monitor the change in $[Zn^{2+}]i$ or $[Ca^{2+}]i$, cells were pretreated with 1 µM FluoZin-3-AM [17] or 1 µM Fluo-3-AM [18] for at least 60 min prior to experiments. FluoZin-3 or

Fluo-3 fluorescence of intact living cells only was measured by flow cytometry, using the CytoACE-150 software package (JASCO, Tokyo, Japan). Dead cells and cells with deteriorating membranes were stained with propidium iodide such that cells exhibiting propidium iodide fluorescence were excluded from this measurement. The excitation wavelength for all fluorescent probes was 488 nm and emission wavelengths used for detection were 600 ± 20 nm for propidium (red fluorescence) and 530 ± 20 nm for Fluo-3 and FluoZin-3 (green fluorescence).

Experimental limitation and statistical analyses

There is an experimental limitation in rat thymocytes. Apoptosis spontaneously occurs at 5–6 h after the dissection of thymus because cell shrinkage, an early maker of apoptosis, is observed. Therefore, the main experiment should be completed within 3 h after the start of drug treatment. It is quite difficult to examine the effect of drugs when the cells are treated with the drugs for 4 h or longer.

Statistical analyses were performed using Tukey's *post-hoc* test and/or a paired t-test. *P*-values < 0.05 were considered statistically significant. Data show the mean ± standard deviation of 4–5 independent experiments. Each series of experiments was conducted in duplicate, unless otherwise specified.

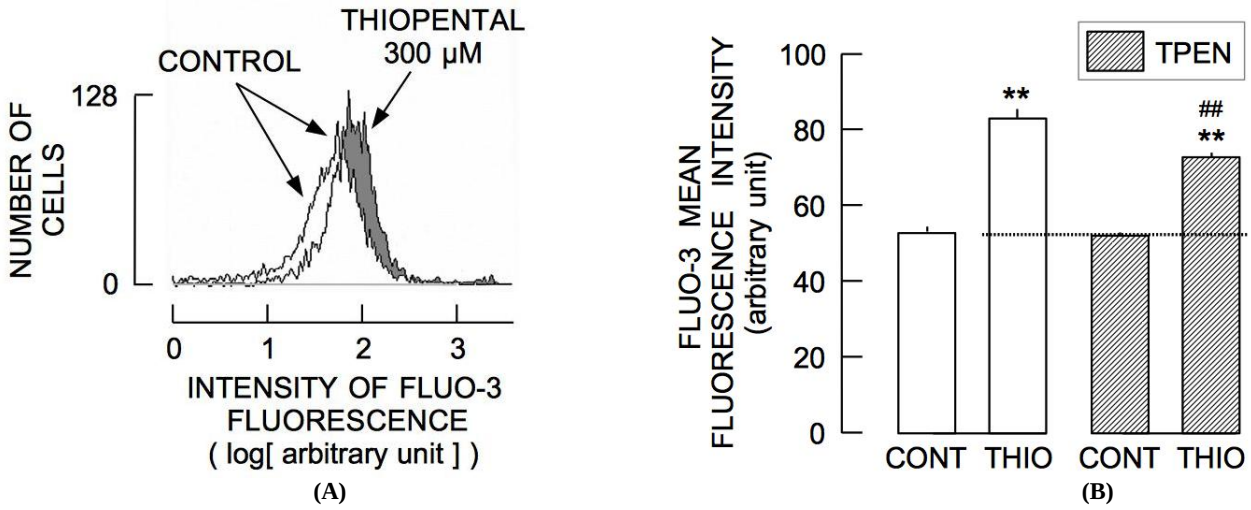

Figure 1. Thiopental-induced augmentation of Fluo-3 fluorescence. (A) Fluo-3 fluorescence histogram shift induced by 300 μM thiopental. Histograms were constructed using 3000 cell events in the absence (CONTROL) and presence (THIOPENTAL) of thiopental. (B) TPEN-induced attenuation of Fluo-3 fluorescence augmented by thiopental. Columns and bars respectively indicate mean intensity of Fluo-3 fluorescence and standard deviation (four samples) in the absence (CONT) and presence (THIO) of 300 μM thiopental. The right panel column-pair (filled columns) was obtained from cells in the presence of TPEN. Asterisks (**) indicate a significant difference ($P < 0.01$) between the control (CONT) and thiopental (THIO) groups. Pound signs (##) indicate a significant difference ($P < 0.01$) between cells treated with thiopental in the absence or presence of TPEN.

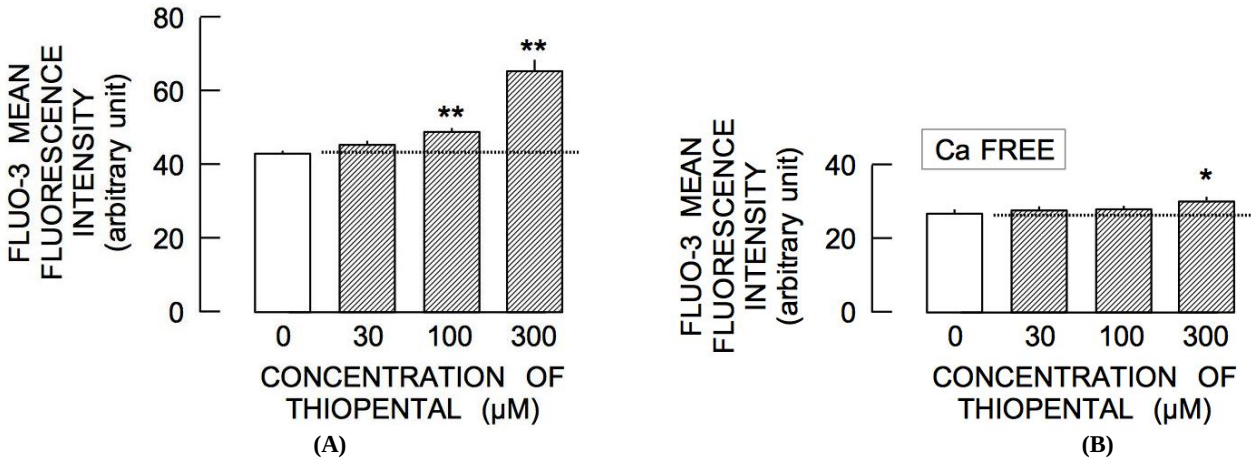

Figure 2. Changes in Fluo-3 fluorescence in response to increasing doses of thiopental in the cells treated with TPEN. (A) Changes by thiopental under the Ca^{2+}-containing control condition (2 mM $CaCl_2$). (B) Changes by thiopental under the Ca^{2+}-free condition (2 mM MgCl2 and 300 μM EDTA). Columns and bars respectively indicate mean intensity of Fluo-3 fluorescence and standard deviation (four samples) in the absence (empty column) and presence (filled columns) of thiopental. Asterisks (*, **) indicate a significant difference ($P < 0.05, 0.01$) between the control and thiopental-treated groups.

■ Results and Discussion

We examined the effect of thiopental on intracellular Ca^{2+}levels in rat thymic lymphocytes. The Fluo-3 fluorescence was measured every 10 min after the start of application of thiopental. The intensity of Fluo-3 fluorescence time-dependently increased and the steady-state intensity attained with 30–40 min after the application of thiopental. The effect of thiopental was examined at 1 h after the start of drug application. Treatment of cells with 300 μM thiopental for 1 h shifted the histogram of Fluo-3 fluorescence, an indicator of intracellular Ca^{2+}, towards a higher mean intensity (Figure 1A). Such a histogram shift indicates that all cells responded to thiopental with augmentation of Fluo-3 fluorescence. As shown in Figure 1B, the augmentation of Fluo-3 fluorescence by thiopental was partially, but significantly, attenuated in the presence of 10 μM TPEN, which greatly diminished FluoZin-3 fluorescence.

Moreover, we examined the effect of thiopental-induced augmentation of Fluo-3 fluorescence in the presence and absence of external Ca^{2+}. Fluo-3 fluorescence augmentation by thiopental consisted of TPEN-sensitive and -insensitive components. Therefore, to negate the contribution of intracellular Zn^{2+} to Fluo-3 fluorescence, the change in Fluo-3 fluorescence by thiopental was examined in the presence of 10 μM TPEN. Treatment of cells with 100–300 μM thiopental for 1 h significantly increased mean intensity of Fluo-3 fluorescence under normal Ca^{2+} conditions (Figure 2A). This was not the case for 30 μM thiopental. Thiopental-induced augmentation was greatly attenuated under Ca^{2+}-free conditions (substitution of $CaCl_2$ with equimolar $MgCl_2$, as well as addition of 300 μM EDTA) (Figure 2B). (Figure 2 near here). This increase in $[Ca^{2+}]i$ is mainly due to increased influx of extracellular Ca^{2+}, given that the removal of external Ca^{2+} greatly suppresses the response elicited by thiopental. Therefore, thiopental may increase membrane Ca^{2+} permeability.

Thiopental-induced augmentation effect of FluoZin-3 fluorescence in the presence and absence of external Zn^{2+} examined in rat thymic lymphocytes. Augmentation of Fluo-3 fluorescence by thiopental possessed a TPEN-sensitive component as described above. Tyrode's solution contains small amounts (approximately 230 nM) of zinc deriving from the composition of salts, as well as reagent preparation [19]. Thiopental-induced fluctuations in FluoZin-3 fluorescence (an indicator of intracellular Zn^{2+}) were examined under nominally Ca^{2+}-free conditions (substitution of $CaCl_2$ with equimolar $MgCl_2$). The FluoZin-3 fluorescence was also measured every 10 min after the start of

application of thiopental. The intensity of FluoZin-3 fluorescence time-dependently increased and the steady-state intensity attained with 30–40 min after the application of thiopental. Treatment of cells with 300 µM thiopental for 1 h slightly, but significantly, increased intensity of FluoZin-3 fluorescence in both the absence and presence of 10 µM DTPA (which chelates external Zn^{2+}). Augmentation of FluoZin-3 fluorescence by thiopental appears to be less dependent on external Zn^{2+} than on external Ca^{2+}. However, addition of 3 µM $ZnCl_2$ to the cell suspension enhanced the thiopental-induced alteration in FluoZin-3 fluorescence (Figure 3B). Such elevation in extracellular Zn^{2+} concentration greatly increased the steady-state level of FluoZin-3, and magnified the thiopental-induced increase in FluoZin-3 fluorescence intensity. Thus, external Zn^{2+} appears to contribute to augmentation of FluoZin-3 fluorescence by thiopental**.** Thiopental at 300 µM may generally increase thymic lymphocyte membrane permeability for metal divalent cations. In addition, thiopental increases $[Ca^{2+}]i$ under Ca^{2+}-free extracellular conditions (Figure 2), and $[Zn^{2+}]i$ under Zn^{2+}-free extracellular conditions (Figure 3). Therefore, thiopental mobilizes intracellular Ca^{2+} and Zn^{2+}. It is reported that thiopental induces Ca^{2+} release from intracellular calcium stores in myocytes [20, 21] and increases lymphocyte oxidative stress [22]. Oxidative stress causes an interchange of zinc-thiol/disulfide that releases Zn^{2+} [23].

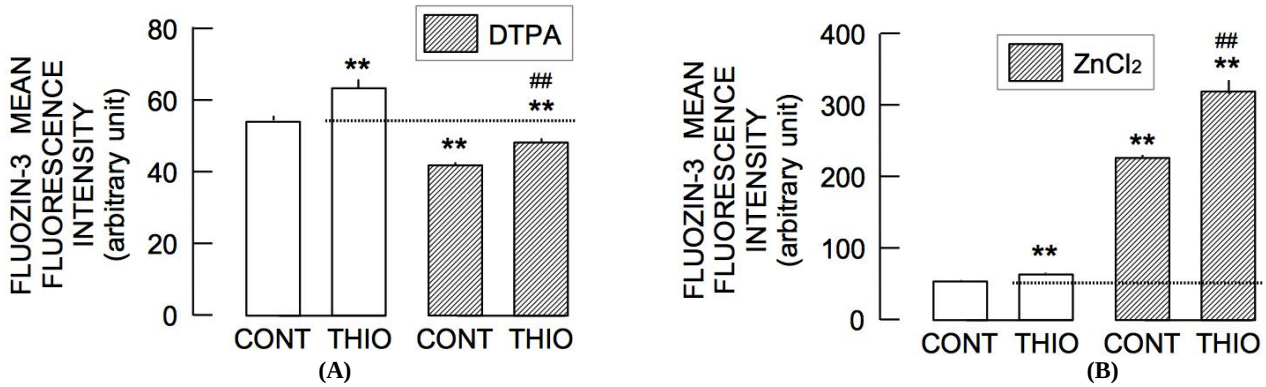

Figure 3. Thiopental-induced augmentation of FluoZin-3 fluorescence. (A) Thiopental-induced augmentation of FluoZin-3 fluorescence in the absence (empty columns) and presence (filled columns) of DTPA. Columns and bars respectively indicate mean intensity of FluoZin-3 fluorescence and standard deviation (four samples) in the absence (CONT) and presence (THIO) of 300 µM thiopental. Asterisks (**) indicate a significant difference ($P < 0.01$) between the control (CONT, left panel) and other groups. Hashtag signs (##) indicate a significant difference ($P < 0.01$) between control (CONT) and thiopental-treated (THIO) cells in the presence of DTPA. (B) Thiopental-induced augmentation of FluoZin-3 fluorescence in the presence of 3 µM ZnCl2. Asterisks (**) indicate a significant difference ($P < 0.01$) between the control (CONT, left panel) and other groups. Hashtag signs (##) indicate a significant difference ($P < 0.01$) between control (CONT) and thiopental-treated (THIO, right panel) cells in the presence of ZnCl2.

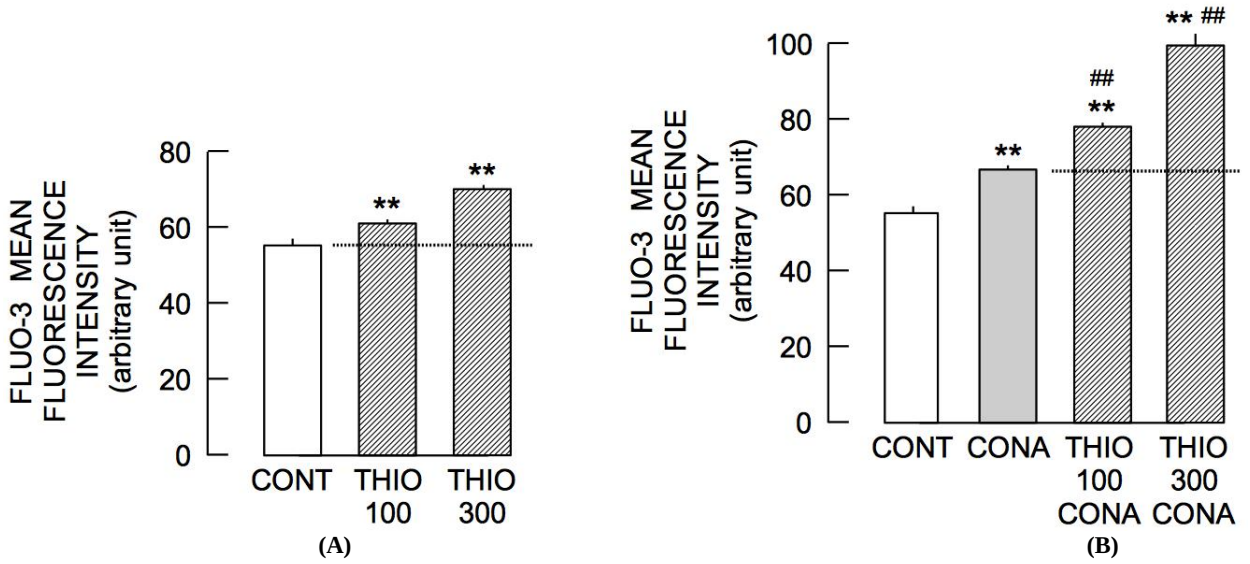

Figure 4. Thiopental-induced augmentation of Fluo-3 fluorescence in the absence (upper panel) and presence (lower panel) of concanavalin A. Columns and bars respectively indicate mean intensity of Fluo-3 fluorescence and standard deviation (four samples). Asterisks (**) indicate a significant difference ($P < 0.01$) between the control (CONT, left panel) and other groups. Hashtag signs (##) indicate a significant difference ($P < 0.01$) between control and thiopental-treated cells in the presence of concanavalin A.

We studied the effect of Fluo-3 fluorescence in concanavalin A-activated lymphocytes by thiopental. Concanavalin A has been in use for many years to study lymphocyte immunomodulation [24]. It is well known that lymphocytes respond to the mitogen concanavalin A by increasing $[Ca^{2+}]i$ [25]. To examine whether thiopental affects the concanavalin A-induced $[Ca^{2+}]i$ response, cells were simultaneously incubated with 100–300 µM thiopental and 10 µg/mL concanavalin A. The application of concanavalin A also increased the intensity of Fluo-3 fluorescence and the steady state attained within 20 min after the application. This result is similar to that previously reported by O'Flynn et al. [25]. The combination of concanavalin A and thiopental further increased the intensity of Fluo-3 fluorescence (Figure 4). Fluo-3 fluorescence increase by thiopental in the presence of concanavalin A was more than twice that induced by thiopental alone.

We showed that thiopental increases both $[Ca^{2+}]i$ and $[Zn^{2+}]i$ in quiescent thymic lymphocytes. Because both Ca^{2+} and Zn^{2+} are lymphocyte intracellular signaling messengers [7-10], thiopental may disturb some lymphocyte functions. Concanavalin A has been in use as a mitogen for many years [24], and lymphocytes respond to this molecule by increasing $[Ca^{2+}]i$ [25]. Thiopental and concanavalin A synergize to produce a greater increase in lymphocyte $[Ca^{2+}]i$ (i.e., the thiopental-induced increase in $[Ca^{2+}]i$ is enhanced in activated lymphocytes). Since an excessive increase in $[Ca^{2+}]i$ causes thymic lymphocyte apoptosis [11, 14], higher-dose thiopental has the potential to induce apoptosis. As to the thiopental-induced increase in $[Zn^{2+}]i$, Zn^{2+} is reported to exert a dose-dependent opposite action on apoptosis in murine thymocytes [12]. Furthermore, there are contradictory papers on the action of thiopental on lymphocyte apoptosis [15, 16]. During the anesthetic use of thiopental, plasma concentrations reach a maximum of between 60 and 80 µg/ml in the majority of cases [26]. These levels are equivalent to molecular concentrations of 227–303 µM, which would thus be sufficient to impact lymphocyte cellular functions. Therefore, in this aspect, further studies are necessary.

Conclusion

We investigated the effect of thiopental on intracellular Ca^{2+} ($[Ca^{2+}]i$) levels using a flow-

cytometric technique, rat thymic lymphocytes. Treatment of cells with 300 μM thiopental increased the $[Ca^{2+}]i$ and $[Zn^{2+}]i$ level. Under intracellular Zn^{2+}-free conditions, 100–300 μM thiopental was still able to induce a statistically significant increase in $[Ca^{2+}]i$, whereas removal of extracellular Ca^{2+} greatly reduced the increase in $[Ca^{2+}]i$ induced by this dose of thiopental. The thiopental-induced increase in $[Ca^{2+}]i$ was mainly due to an increased influx of Ca^{2+}. Moreover, the thiopental-induced elevation of $[Zn^{2+}]i$ was due to an increase in both influx of Zn^{2+} and intracellular Zn^{2+} release. The results of the present study provide information for using thiopental in a safe manner.

Acknowledgments

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Conflicts of interest

The authors declare no conflicts of interest.

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