Research Square

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Increased Intracellular Zn^{2+} Levels Suppress Ca^{2+}-Dependent Cytotoxicity in Rat Thymocytes

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Abstract

Zinc is an essential trace element that plays catalytic, structural, and regulatory roles in cellular physiology. Intracellular free Zn^{2+} participates in various signaling pathways, including the regulation of cell survival and death. However, its role in Ca^{2+}-dependent cytotoxicity remains unclear. We investigated the involvement of intracellular free Zn^{2+} in Ca^{2+}-dependent cell death using rat thymocytes. In cells treated with the ionophore A23187, cell death was evaluated using propidium iodide staining, intracellular Ca^{2+} levels were assessed using Fluo-3 fluorescence, and membrane potential changes were monitored using oxonol fluorescence. Simultaneous treatment with low concentrations of Zn^{2+} (1–10 μM) significantly suppressed A23187-induced cell death, whereas extracellular Zn^{2+} chelation with DTPA did not attenuate cytotoxicity, indicating that extracellular Zn^{2+} is not required for A23187-induced cell death. The protective effect of Zn^{2+} was strongly dependent on the timing of administration and was most prominent when Zn^{2+} was added concurrently with A23187, gradually diminishing when Zn^{2+} addition was delayed. Zn^{2+} significantly suppressed the A23187-induced increase in intracellular Ca^{2+} concentration, and this effect was reversed by the Zn^{2+} chelator TPEN. Thus, Zn^{2+} is essential for the modulation of Ca^{2+} dynamics. Zn^{2+} attenuated A23187-induced membrane hyperpolarization, suggesting suppression of Ca^{2+}-dependent membrane excitability. These findings indicate that intracellular Zn^{2+} acts as a negative regulator of early Ca^{2+}-dependent cytotoxicity by suppressing intracellular Ca^{2+} elevation and membrane hyperpolarization in rat thymocytes. In conclusion, this study suggests that intracellular Zn^{2+} may act as a negative regulator of early-phase Ca^{2+}-dependent cytotoxicity in rat thymocytes, although the detailed underlying mechanisms remain to be elucidated.

Introduction

Zinc is an essential trace element in the human body, functioning as a catalytic, structural, and regulatory ion. Adults have 1.4–2.3 g of zinc widely distributed across tissues, with about 85% localized in the muscle. Zinc deficiency leads to diverse physiological abnormalities, including impaired immune function, skin disorders, growth retardation, and neurological dysfunction (Prasad 2008; Stephan C 2022). At the cellular level, zinc metabolism is critical for numerous biological processes, and the disruption of zinc homeostasis is frequently associated with disease progression (Murakasmi and Hirano 2008; Fukada et al. 2011). Zinc binds to approximately 3,000 proteins, with nearly 10% serving as enzyme cofactors. It is indispensable for zinc finger motifs, which are involved in transcriptional regulation, and contributes to enzymatic substrate specificity (Kimura and Kambe 2016).

Although cytosolic free zinc is maintained at picomolar concentrations, total intracellular zinc levels reach 200–300 μM. Intracellular zinc homeostasis is primarily regulated by two transporter families—SLC30 (ZnT), which mediates zinc efflux, and SLC39 (ZIP), which mediates zinc influx—as well as metallothioneins, which serve as major zinc storage proteins. Various stimuli induce transient increase in intracellular free Zn^{2+}. Extracellular signals such as cytokines, growth factors, and hormones modulate ZnT and ZIP transporter activity, while intracellular organelles, including the endoplasmic reticulum and mitochondria, can rapidly release zinc into the cytosol, generating so-called zinc waves that often occur

in concert with Ca^{2+} signaling (Yamasaki et al. 2007). Oxidative stress also promotes zinc release from metallothionein, markedly elevating free Zn^{2+} levels. Increased extracellular zinc enhances zinc influx via ZIP transporters, and physiological events such as fertilization and cell-cycle progression involve transient zinc elevations, exemplified by the zinc spark in oocytes (Que et al. 2015).

Recently, increasing attention has been directed toward the role of free Zn^{2+} in the regulation of cell death. Although Zn^{2+} inhibits apoptosis by suppressing execution factors such as caspase-3 and protein tyrosine phosphatases (Yamasaki et al. 2007), elevated Zn^{2+} promotes cell death through the dysregulation of intracellular Ca^{2+} homeostasis. Thus, whether Zn^{2+} predominantly suppresses or enhances cell death remains controversial (Que et al. 2015). Rapid increases in intracellular Ca^{2+} and Zn^{2+} have also been linked to cytotoxicity induced by various chemical agents, and accumulating evidence indicates that Zn^{2+} modulates apoptosis in a Ca^{2+}-dependent manner, although the underlying molecular mechanisms remain unclear.

Increasing evidence has highlighted the relationship between zinc and immune cell function. Thymocytes predominantly consist of immature T cells, which are highly susceptible to cell death during immunological selection. Therefore, in this study, we investigated intracellular free Zn^{2+} in Ca^{2+}-dependent cytotoxicity and the relationship between zinc and cell death using rat thymocytes.

Methods

Fluorescent Probes and Chemicals

Propidium iodide (PI) was used to assess cell death. Fluo-3-AM was used to study changes in intracellular Ca^{2+} levels (Okazaki et al. 1996). FluoZin-3 was used to study changes in intracellular Zn^{2+} levels (Kimura et al. 2011; Oyama et al. 2020). These probes were obtained from Invitrogen and Thermo Fisher Scientific Inc. (Eugene, OR, USA). Membrane-impermeable and -permeable Zn^{2+} chelators, diethylenetriamine-N,N,N′,N″,N‴-pentaacetic acid (DTPA), N,N,N′,N′-tetrakis (2-pyridylmethyl)ethylenediamine (TPEN), and Cell Counting Kit-8 were purchased from Dojin Chemicals (Kumamoto, Japan). A23187 and $ZnCl_2$ were obtained from Sigma Aldrich (St. Louis, MO, USA). Thapsigargin and other chemicals were purchased from Wako Pure Chemicals (Osaka, Japan).

Cell Suspension

The use of experimental animals in this study was approved by the ethical committees of Tokushima University (Tokushima, Japan), Tokushima Bunri University (Tokushima, Japan), and Sonoda University (Hyogo, Japan) under registration numbers T29-52, H30-3, and 2026-1, respectively.

Thymocytes were selected for the chemical cytotoxicity experiment because cells with intact plasma membranes can be isolated from tissues without enzymatic digestion, and the majority of thymocytes are T cells. However, thymocytes are susceptible to spontaneous apoptosis during extended incubation.

Since cell shrinkage is one of the earliest morphological changes associated with apoptosis, all experiments were completed within 6–7 h after organ dissection to minimize the influence of spontaneous apoptosis (Bortner and Cidlowski 1998).

Cell suspensions were prepared from thymus glands. Briefly, thymus glands were dissected from 8- to 12-week-old male Wistar rats (n = 8) obtained from CLEA Japan (Tokyo, Japan) and anesthetized with 50 mg/kg i.p. thiopental sodium (Ravonal, Mitsubishi Tanabe Pharma, Osaka, Japan). The glands were sliced with a razor, and the slices were triturated in chilled Tyrode's solution (150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM glucose) to dissociate individual lymphocytes. The pH of the solution was adjusted to 7.3–7.4 with 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and an appropriate amount of NaOH. The solution containing dissociated cells was passed through a mesh (diameter: 25 μm) to prepare the cell suspension (5×10^5 cells/mL). Cells were incubated at 36–37°C for 1 h before experimental use.

Fluorescence Measurements of Cellular Parameters

Fluo-3-AM, FluoZin-3, DTPA, A23187, and TPEN were initially dissolved in dimethyl sulfoxide (DMSO). DMSO at final concentrations of 0.1–0.3% did not induce cell death. All experiments using the cell suspension were performed at 36–37°C. Cell death induced by Ca^{2+} influx was elicited using the calcium ionophore A23187 (100 nM). $ZnCl_2$ was added to the cell suspension to achieve final concentrations of 1–10 μM, based on previous studies reporting that $ZnCl_2$ treatment increases intracellular Zn^{2+} levels (Kimura et al. 2011; Oyama et al. 2020). In the present study, changes in intracellular Zn^{2+}-associated fluorescence were also assessed using FluoZin-3. Oxidative stress was induced using 100 μM H_2O_2. The control group consisted of cells treated with vehicle alone (DMSO) without any additional treatment. Cellular and membrane parameters were measured using fluorescent probes and a flow cytometer equipped with software for data collection and analysis (CytoACE-150; JASCO, Tokyo, Japan, version 3.06). PI (5 μM) was used to assess cell death, and PI-positive cells were considered dead. The fluorescence of Fluo-3 and FluoZin-3 was measured only in PI-negative cells. To monitor changes in Fluo-3 fluorescence associated with intracellular Ca^{2+}, cells were incubated with 500 nM Fluo-3-AM for 1 h before fluorescence measurements. To monitor changes in FluoZin-3 fluorescence associated with intracellular Zn^{2+}, cells were incubated with 1 μM FluoZin-3-AM for 30 min before fluorescence measurements. The excitation and emission wavelengths were 488 nm and 530 ± 15 nm, respectively, for both Fluo-3 and FluoZin-3. For PI, the excitation and emission wavelengths were 488 nm and 600 ± 20 nm, respectively.

Cell viability assay

For the cell viability assay, thymocytes were seeded in triplicates into 96-well plates (1×10^4 cells per well). Thymocytes were treated with thapsigargin and the indicated concentrations of $ZnCl_2$ at 37°C for 3

h. Cell viability was assessed using a Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's protocol.

Statistical Analysis

Statistical analysis was performed using Excel Toukei 2010 (Social Survey Research Information Co., Ltd. Tokyo, Japan). Data were analyzed using ANOVA with post-hoc Tukey's multivariate analysis. P-value < 0.05 was considered significant. Data are reported as mean ± standard deviation of four to eight experiments.

Results

Intracellular Zn^{2+} Suppresses Ca^{2+}-Dependent Cell Death

The relationship between Ca^{2+} influx-induced cell death and Zn^{2+} was examined. To this end, the effects of 1–10 μM Zn^{2+} on cells treated with A23187 were investigated. The effect of 3 μM $ZnCl_2$ treatment on intracellular Zn^{2+} levels in thymocytes was first examined using the fluorescent Zn^{2+} indicator FluoZin-3. Treatment with 3 μM $ZnCl_2$ markedly increased FluoZin-3 fluorescence, whereas this increase was attenuated in the presence of DTPA or TPEN (Supplemental Fig. 1). These results confirmed that 3 μM $ZnCl_2$ treatment increased intracellular Zn^{2+} levels in thymocytes. Cells were simultaneously treated with A23187 and Zn^{2+} for 3 h, and cell death was subsequently assessed using PI staining. To further examine the effect of Zn^{2+} on Ca^{2+}-dependent cytotoxicity, we performed additional experiments using thapsigargin as an alternative model of Ca^{2+} overload. Thapsigargin-induced cell death was also suppressed by Zn^{2+} treatment (Supplemental Fig. 2).

Neither treatment with DTPA alone nor with 1–10 μM Zn^{2+} alone produced a significant difference in cell death compared with the control. Simultaneous treatment with A23187 and DTPA for 3 h significantly increased the proportion of PI-positive cells (Fig. 1). In contrast, co-treatment with 1–10 μM Zn^{2+} significantly suppressed the increase in cell death induced by A23187 (Fig. 1). However, Ca^{2+}-dependent cell death increased following co-treatment with A23187 and 10 μM Zn^{2+} compared with that following co-treatment with A23187 and 1–3 μM Zn^{2+}. Therefore, we examined the effect of Zn^{2+} on the susceptibility of rat thymocytes to oxidative stress. Exposure to 100 μM H_2O_2 for 3 h significantly increased cell death, and co-treatment with 10 μM Zn^{2+} further increased H_2O_2-induced cell death (Supplemental Fig. 3). These results suggest that although Zn^{2+} suppresses A23187-induced Ca^{2+}-dependent cell death, 10 μM Zn^{2+} may increase susceptibility to cell death under oxidative stress conditions.

Taken together, these results suggest that Zn^{2+} suppresses A23187-induced Ca^{2+}-dependent cell death. Conversely, 10 μM Zn^{2+} increases susceptibility to cell death under oxidative stress conditions, suggesting a potential cytotoxic role for 10 μM Zn^{2+} in environments dominated by reactive oxygen species. Based on these observations, 3 μM Zn^{2+} was used for subsequent experiments.

Elevation of Intracellular Zn^{2+} Suppresses Ca^{2+}-Dependent Cell Death in the Early Phase

The relationship between the timing of Zn^{2+} addition and A23187-induced cytotoxicity was examined. Treatment with A23187 significantly increased cell death compared with the control group, whereas treatment with 3 μM Zn^{2+} alone did not significantly affect cell death. Simultaneous addition of 3 μM Zn^{2+} with A23187 significantly suppressed A23187-induced cell death. When Zn^{2+} was added 1 h after A23187 stimulation, no significant suppression of cell death was observed, whereas addition of Zn^{2+} 2 h after A23187 stimulation significantly reduced cell death. These results indicate that Zn^{2+} suppressed A23187-induced cytotoxicity when added simultaneously with A23187 or 2 h after A23187 stimulation (Fig. 2).

TPEN Abolished the Suppressive Effect of Zn^{2+} on Ca^{2+}-Dependent Cell Death

The effects of Zn^{2+} on intracellular Ca^{2+} levels in rat thymocytes were examined based on Fluo-3 fluorescence. Exposure of cells to A23187 alone resulted in a significant increase in Fluo-3 fluorescence intensity (Fig. 3). Simultaneous treatment with A23187 and Zn^{2+} significantly suppressed the increase in Fluo-3 fluorescence compared with that after treatment with A23187 alone. Furthermore, when A23187, Zn^{2+}, and TPEN were added simultaneously, the increase in Fluo-3 fluorescence was significantly restored compared with the condition without TPEN. These results indicate that Zn^{2+} suppresses the A23187-induced elevation of intracellular Ca^{2+} in rat thymocytes.

Zn^{2+} Suppresses Ca^{2+}-Dependent Membrane Hyperpolarization

A23187 and Zn^{2+} induced membrane hyperpolarization in rat thymocytes, as assessed by oxonol fluorescence measurements, suggesting the involvement of Ca^{2+}-activated K^+ channels. Treatment with Zn^{2+} alone did not induce a significant change in oxonol fluorescence intensity (Fig. 4). In contrast, A23187 alone significantly decreased oxonol fluorescence, indicating the induction of membrane hyperpolarization. Simultaneous treatment with A23187 and Zn^{2+} significantly attenuated the decrease in oxonol fluorescence compared with that after treatment with A23187 alone. Furthermore, when A23187, Zn^{2+}, and TPEN were added simultaneously, the decrease in oxonol fluorescence was again significantly restored compared with the condition without TPEN. These results suggest that Zn^{2+} suppresses A23187-induced membrane hyperpolarization.

Discussion

Dynamic changes in intracellular free zinc have been implicated in diverse cellular signaling pathways and in the maintenance of cellular homeostasis (Yamasaki et al. 2007; Que et al. 2015). However,

although the involvement of zinc in cell death has been reported, the role of intracellular free Zn^{2+} in cell death remains poorly understood. Therefore, we investigated the involvement of intracellular free Zn^{2+} in the regulation of cell death.

In this study, A23187-induced cell death in rat thymocytes was significantly attenuated following co-administration of Zn^{2+} with A23187. However, zinc treatment alone induced cell death, although the effect was not statistically significant. In addition, treatment with DTPA, a membrane-impermeable metal chelator that sequesters extracellular free Zn^{2+}, enhanced A23187-induced cell death. Therefore, an increase in intracellular free zinc may selectively suppress A23187-induced cell death. A23187 is a calcium ionophore that induces an increase in intracellular Ca^{2+} concentration, and its mechanism of action involves two major pathways (Reed and Hardy 1972). These pathways include the influx of extracellular Ca^{2+} into the cell and the release of Ca^{2+} from intracellular stores, such as the endoplasmic reticulum and mitochondria. The elevation of intracellular Ca^{2+} concentration induced by these mechanisms has been clearly demonstrated to trigger cell death, including apoptosis and necrosis (Trump and Berezesky 1995). In addition, the inhibitory effect of Zn^{2+} on cell death was also observed under thapsigargin treatment, suggesting that the protective effect of Zn^{2+} is not restricted to A23187-induced Ca^{2+} overload. These findings suggest that the inhibitory effect of Zn^{2+} may be associated with the suppression of Ca^{2+}-dependent cell death rather than being specific to A23187-induced cytotoxicity.

Furthermore, we examined how zinc influences intracellular Ca^{2+} dynamics. Measurements of intracellular Ca^{2+} levels using Fluo-3 revealed that the A23187-induced increase in cytosolic Ca^{2+} concentration was significantly reduced in the presence of Zn^{2+}. This effect was abolished by the Zn^{2+} chelator TPEN, indicating that Zn^{2+} itself is required for the suppression of Ca^{2+}. Given that A23187 functions as a divalent cation ionophore, it is unlikely that Zn^{2+} directly blocks Ca^{2+} entry across the plasma membrane. Rather, Zn^{2+} may indirectly modulate Ca^{2+} dynamics by influencing membrane potential or the electrochemical driving force for Ca^{2+} influx.

Membrane potential measurements supported this possibility; A23187 induced marked membrane hyperpolarization, which was significantly attenuated in the presence of Zn^{2+}. Rat thymocytes express Ca^{2+}-activated K^+ channels, and Ca^{2+} influx triggered by A23187 is likely to activate these channels, resulting in membrane hyperpolarization (Mahaut-Smith and Mason 1991). Zn^{2+} inhibits voltage-dependent calcium channels, thereby reducing calcium influx (Sun et al. 2007). Zinc suppresses intracellular calcium influx and alleviates calcium overload via the mitochondrial calcium uniporter (Zhao et al. 2022; Guo et al. 2025). Taken together, these findings raise the possibility that Zn^{2+} may influence Ca^{2+}-dependent cellular responses through the modulation of Ca^{2+}-activated K^+ channels, voltage-dependent Ca^{2+} channels, and/or the mitochondrial calcium uniporter. Such modulation could potentially affect intracellular Ca^{2+} dynamics and membrane polarization, thereby contributing to the attenuation of Ca^{2+}-dependent cell death. However, these mechanisms were not directly examined in the present study, and the specific molecular pathways involved remain to be determined.

We also examined the effect of Zn^{2+} addition timing on A23187-induced cell death. When Zn^{2+} was added simultaneously with A23187, A23187-induced cell death was significantly suppressed. However, this inhibitory effect gradually diminished over time, with no significant suppression observed when Zn^{2+} was added 1 h after A23187 treatment. Our results showed that Zn^{2+}-mediated protection is restricted to the early phase of Ca^{2+}-dependent cell death. The results of this study also indicate that the inhibitory effect of Zn^{2+} is dependent on concentration as well as timing. In our study, A23187-induced cell death increased following A23187 co-treatment with 10 μM Zn^{2+}, compared to that following A23187 co-treatment with 1 or 3 μM Zn^{2+}. Furthermore, under oxidative stress, 10 μM Zn^{2+} appeared to enhance cellular susceptibility to cell death. A previous study reported that excessive Zn^{2+} induces cell death (Abe et al. 2006; Ma, Zhao, and Zu 2015). Specifically, low concentrations of Zn^{2+} (≤ 60 μM) contribute to enhanced cell proliferation and viability, whereas high concentrations (> 80 μM) induce cellular dysfunction. These findings suggest that Zn^{2+} is a bifunctional factor capable of influencing both cell survival and cell death, and its physiological effects are strongly dependent on intracellular concentration and the phase of action. However, the zinc concentration that induced the inhibitory effect on cell death in this study was relatively low, substantially lower than that reported in previous studies (Que et al. 2015). This represents a major finding of this study and warrants further investigation. Furthermore, the Zn^{2+} results are consistent with previous reports indicating that zinc can inhibit Ca^{2+}-dependent cell death (Kitamura et al. 2006). Therefore, these findings strongly suggest that Zn^{2+} is involved in A23187-induced cell death and in the regulation of a broader range of Ca^{2+}-dependent cell death processes. The regulation of intracellular Zn^{2+} dynamics may contribute to the physiological machinery governing the induction and control of cell death.

In this study, the effects of Zn^{2+} were examined using A23187-induced cell death as a model of Ca^{2+}-dependent cytotoxicity. Future studies should determine whether the protective effects of Zn^{2+} are specific to Ca^{2+}-dependent cell death or extend to other forms of cell death induced by distinct cellular stress pathways. In addition, further studies are required to systematically elucidate Zn^{2+} dynamics under physiological conditions and clarify the molecular mechanisms underlying its effects on cell death.

Overall, this study demonstrates that Zn^{2+} suppresses Ca^{2+}-dependent cell death in rat thymocytes, accompanied by a reduction in A23187-induced membrane hyperpolarization. These findings suggest that Zn^{2+} may contribute to the regulation of Ca^{2+}-mediated cell death, at least in part, through modulation of intracellular Ca^{2+} dynamics and membrane excitability. These findings provide evidence for a potential protective role of Zn^{2+} during the early phase of Ca^{2+}-dependent cell death. However, whether this Zn^{2+}-dependent regulatory mechanism is conserved across different cell types and what molecular mechanisms underlie this effect remain to be determined. Further studies using additional cell systems and experimental models will be required to clarify these mechanisms. Importantly, the precise intracellular Zn^{2+} levels under the experimental conditions were not directly determined in the present study. Therefore, direct quantification of intracellular Zn^{2+} and further investigation of the relationship between intracellular Zn^{2+} levels and Ca^{2+}-dependent cytotoxicity will be important areas for future research.

Declarations

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Author Contributions

Conceptualization: Norio Kamemura; **Methodology:** Norio Kamemura; **Formal analysis and investigation:** Masamichi Aea, Norio Kamemura; **Writing - original draft preparation:**Masamichi Aea; **Writing - review and editing:** Noriko Takashima, Akihiro Yoshie; **Validation:** Yasuyuki Izutani; **Data curation:** Masamichi Aea; **Supervision:** Norio Kamemura.

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Declaration of Competing Interests

The authors declare that they have no competing interests.

Data Availability Statement

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Compliance with Ethical Standards

The use of experimental animals in this study was approved by the ethical committees of Tokushima University (Tokushima, Japan), Tokushima Bunri University (Tokushima, Japan), and Sonoda University (Hyogo, Japan) under registration numbers T29-52, H30-3, and 2026-1, respectively.

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Figures

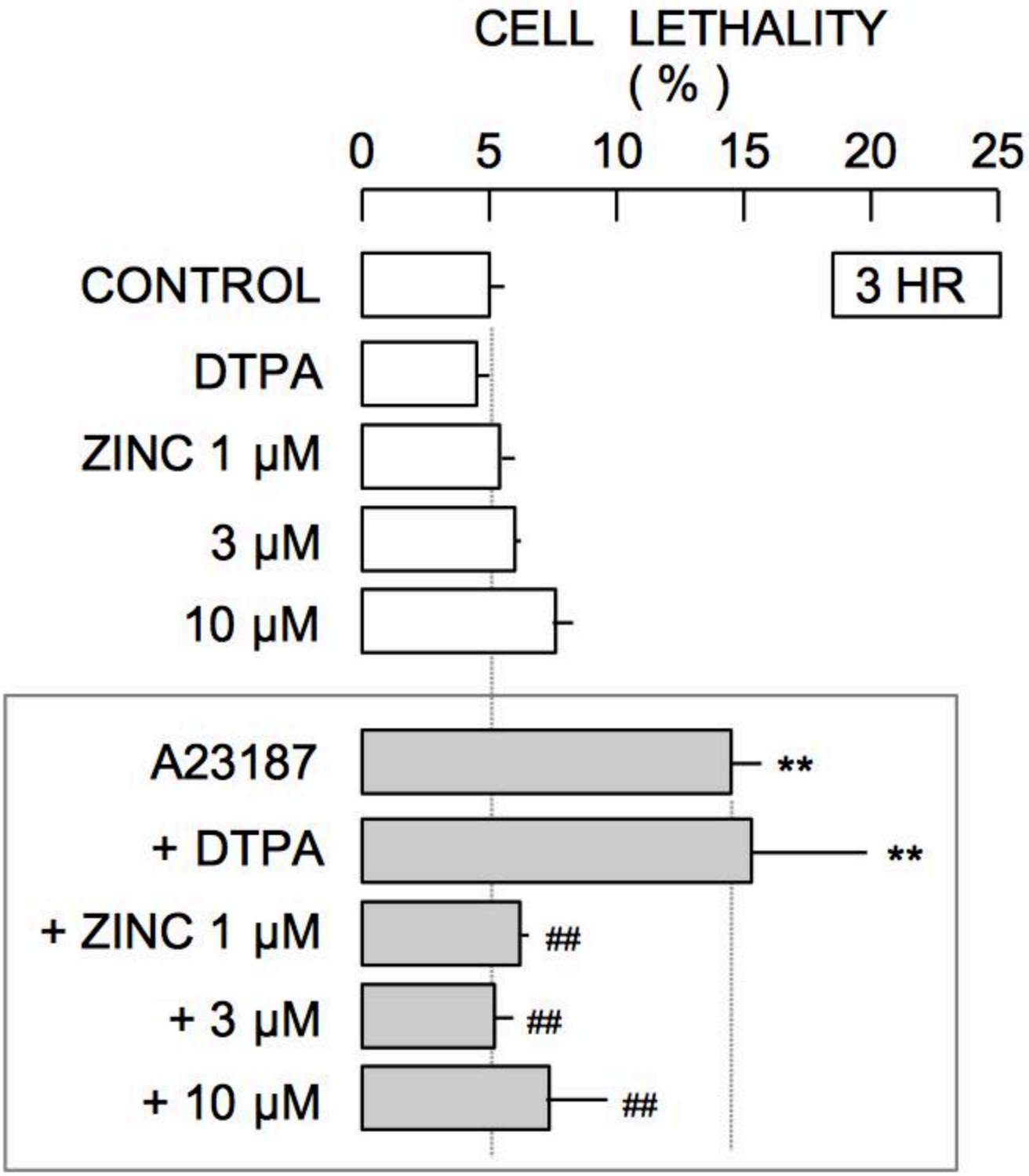


Figure 1

Effects of Zn^{2+} on A23187-induced cell lethality in rat thymocytes. **p < 0.01 between control (0.1% DMSO) and A23187-treated groups. $^{\#\#}p < 0.01$ between positive control groups (A23187 with DTPA) and groups subjected to combined exposure (A23187 with ZINC).

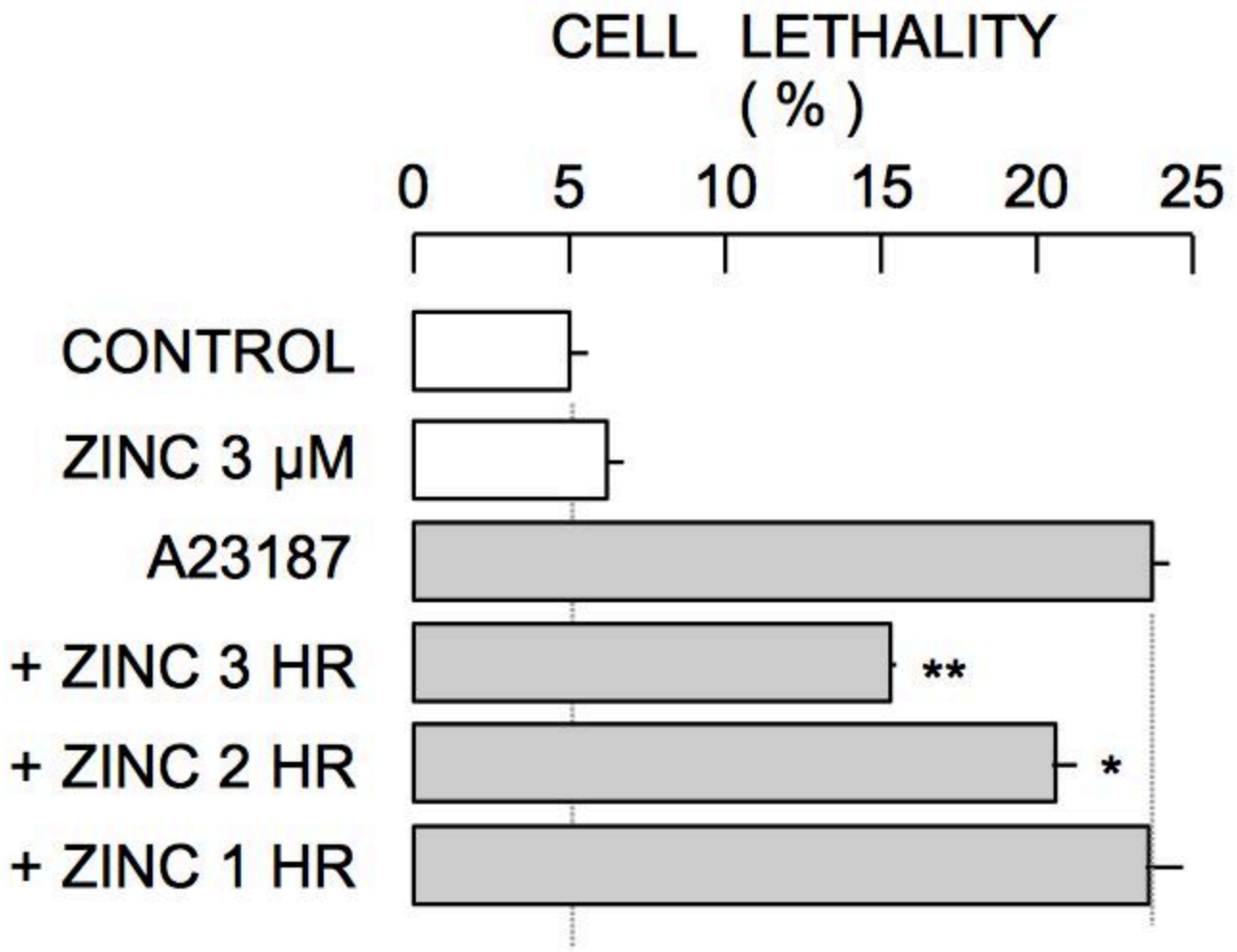


Figure 2

Time-dependent effects of Zn^{2+} on A23187-induced cell lethality. Asterisks (*, **) indicate significant differences ($p < 0.05$, $p < 0.01$) between positive control groups (A23187) and groups subjected to combined exposure (A23187 with zinc).

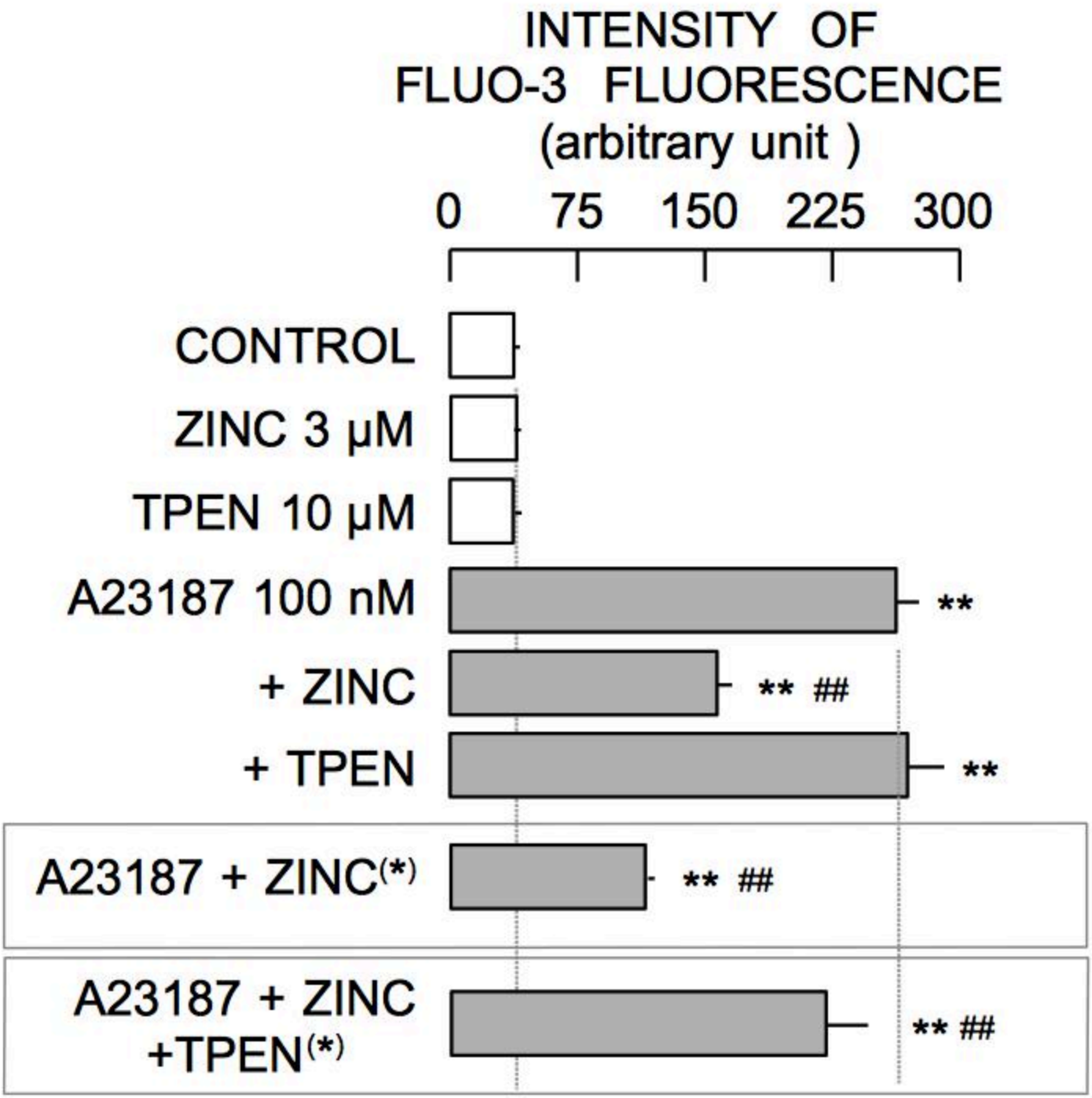


Figure 3

Zinc suppresses the A23187-induced increase in intracellular Ca^{2+} concentration. **p < 0.01 between control (0.1% DMSO) and A23187-treated groups. $^{\#\#}p < 0.01$ between positive control groups (A23187 100 nM) and groups subjected to combined exposure (A23187 with ZINC).

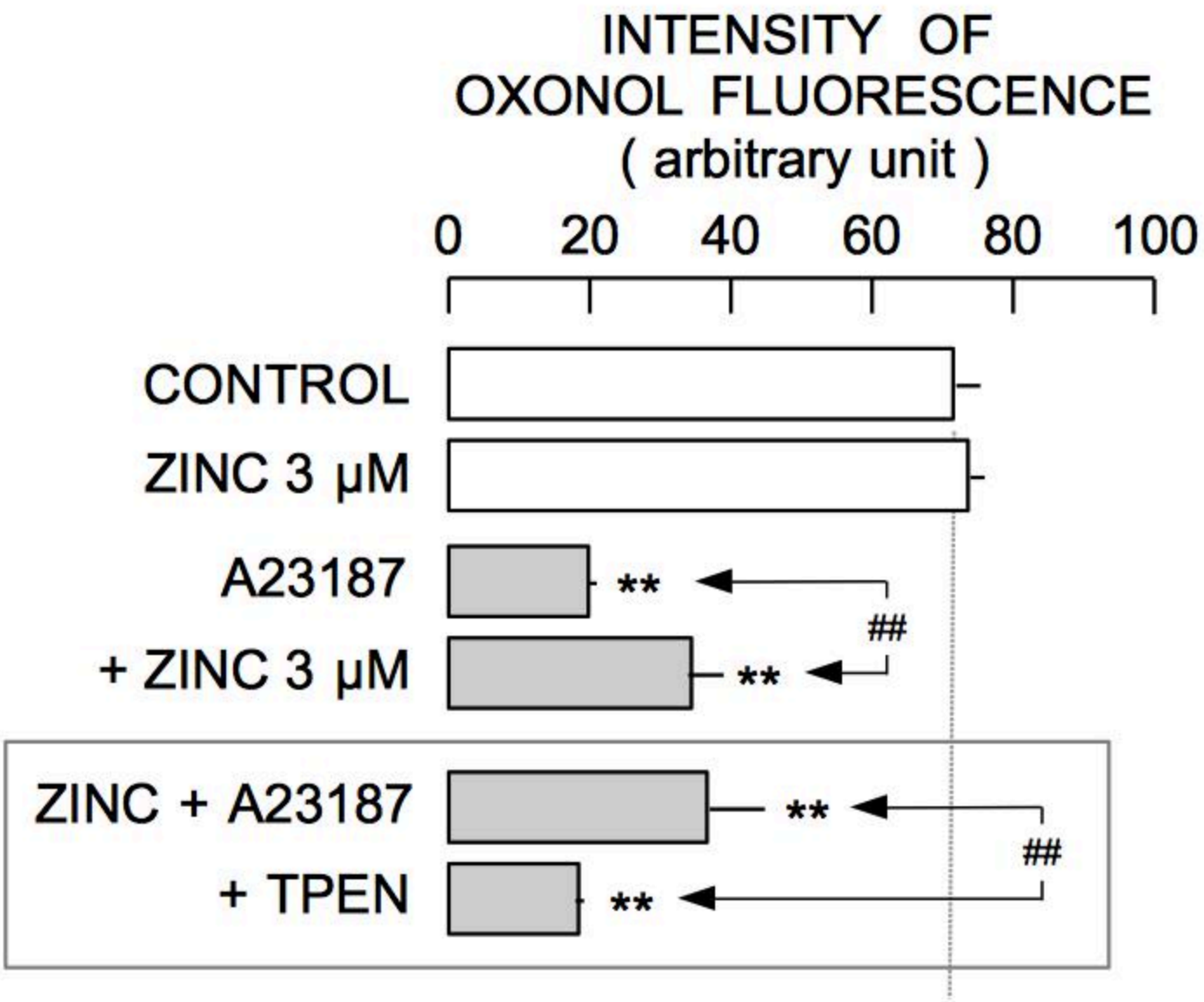


Figure 4

A23187-induced change in the oxonol fluorescence (membrane potential) of living cells. **p < 0.01 between control (0.1% DMSO) and A23187-treated groups. $^{\#\#}p < 0.01$ between positive control groups (A23187) and groups subjected to combined exposure (A23187 with ZINC).

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