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Biochemical and Biophysical Research Communications 335 (2005) 162–167
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Zinc ions suppress mitogen-activated interleukin-2 production in Jurkat cells ☆

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Received 1 July 2005
Available online 25 July 2005

Abstract

Calcineurin (CN) is thought to play an important role in the immune system by regulating cytokine production, for example, interleukin-2 (IL-2) in T-lymphocytes. We have previously shown that physiological concentrations of Zn^{2+} inhibit CN activity in vitro [K. Takahashi, E. Akaishi, Y. Abe, R. Ishikawa, S. Tanaka, K. Hosaka, Y. Kubohara, Zinc inhibits calcineurin activity in vitro by competing with nickel, Biochem. Biophys. Res. Commun. 307 (2003) 64–68], in spite of the fact that Zn^{2+} is an essential element of the CN catalytic domain. In this study, in order to assess whether Zn^{2+} regulates (suppresses) CN activity in vivo and whether Zn^{2+} can be used as an anti-inflammatory and/or immunosuppressive drug, we examined the effects of Zn^{2+} on IL-2 production induced by the mitogen, concanavalin A (ConA), in Jurkat T-cells. Zn^{2+} at 0.2 mM suppressed ConA-induced IL-2 accumulation in the medium of an in vitro culture of Jurkat cells. Zn^{2+} at 0.03–0.3 mM dose-dependently suppressed ConA-induced IL-2 mRNA expression in Jurkat cells. Zn^{2+} also suppressed IL-2 mRNA expression induced by phorbol ester (PMA) and ionomycin. Furthermore, Zn^{2+} and the immunosuppressant FK506 showed an additive inhibitory effect on ConA-induced IL-2 mRNA expression. These results suggest that exogenously added Zn^{2+} may disturb (increase) the intracellular Zn^{2+} concentration and inhibit CN activity, thereby suppressing IL-2 production in Jurkat cells. The present study further indicates that Zn^{2+} may have therapeutic potential in the treatment of T-cell related inflammation and also that Zn^{2+} may be utilized as a supplemental drug with FK506.
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Keywords: Zinc; Calcineurin; Interleukin-2; Jurkat cells

Zn^{2+} is found in a variety of organisms and has been shown to be essential for many cellular functions, such as the actions of some metallo-enzymes, the regulation of gene expression through zinc finger motifs of DNA-binding proteins, and the capsid formation of the human immunodeficiency virus (HIV) [1–8]. Zinc deficiency in humans, thus, results in alterations of many body systems, such as the epidermal, gastrointestinal, central nervous, and immune systems [9–14].

Calcineurin (CN), also known as protein phosphatase 2B, is a Ca^{2+}/calmodulin (CaM)-dependent protein serine/threonine phosphatase that contains Zn^{2+} and Fe^{2+} as essential elements in its catalytic domain [15–18]. CN plays pivotal roles in a variety of cellular functions in both higher and lower eukaryotic organisms. For example, CN is involved in Ca^{2+}-induced nervous cell apoptosis [19] and the hyper-phosphorylation of the tau protein in Alzheimer's disease [20,21]. In T-cell activation, CN functions by de-phosphorylating the nuclear

☆ *Abbreviations:* IL-2, interleukin-2; CN, calcineurin; ConA, concanavalin A; PMA, phorbol 12-myristate 13-acetate; IM, ionomycin.

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doi:10.1016/j.bbrc.2005.07.059

factor of activated T-cells (NFAT) that enhances the expression of interleukin-2 (IL-2) [22]. Two immunosuppressive drugs, cyclosporin A and FK506, have been found to inhibit CN via immunophilins [23–25]. In addition, CN has been shown to be involved in cytokinesis, cell shape, and ion homeostasis in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* [26–28].

While the activity of CN is regulated by Ca^{2+} and CaM in vivo, it can be stimulated by divalent ions, such as Mn^{2+} and Ni^{2+}, in vitro [29–31]. Quite recently, we have found that Zn^{2+} at physiological concentrations inhibits the activity of CN in vitro [32], despite the fact that Zn^{2+} is an essential element for the catalytic domain of CN. It is thus possible that Zn^{2+} is not only an essential element for CN but also a negative regulator of CN activity in T-cells and, thus, of the immune system in vivo.

In this study, in order to assess the above possibility in T-cells and the pharmacological potential of Zn^{2+}, we examined the effects of Zn^{2+} and Zn^{2+} in combination with FK506 on IL-2 production in Jurkat T-cells. We show here that exogenously added Zn^{2+} suppressed the protein production and mRNA expression of IL-2 induced by concanavalin A (ConA: a mitogen). It is also shown that the co-addition of Zn^{2+} and FK506 showed additive inhibitory effects on IL-2 mRNA expression in Jurkat cells. The in vivo role of Zn^{2+} in the regulation of CN activity and the pharmacological potential of Zn^{2+} are also discussed.

Materials and methods

Cell line and reagents. Jurkat cells (human T-lymphocyte line) were used in this study. They were maintained at 37 °C (5% CO_2) in tissue culture dishes filled with a growth medium (an RPMI1640 medium with 10% fetal bovine serum, 25 μg/ml penicillin, and 50 μg/ml streptomycin designated RPMI). All the metal ions used were purchased from Wako Pure Chemical Industries (Osaka, Japan). Phorbol 12-myristate 13-acetate (PMA) and ionomycin (IM) were purchased from Sigma (St. Louis, MO) and stored as 1 mM solutions in dimethyl sulfoxide (DMSO). FK506 was from Calbiochem (San Diego, CA) and stored as 1 μM–1 mM solutions in ethanol (EtOH).

Assay for IL-2 production and cell viability in Jurkat cells. Jurkat cells were pre-incubated in 90-mm culture dishes filled with 10 ml RPMI (at 1×10^6 cells/ml) in the presence or absence of FK506 (1 μM) or $ZnSO_4$ (0.2 mM) for several minutes. ConA (final concentration; 25 μg/ml) was then added to the media, and cells were further incubated for 3, 6, and 12 h. Aliquots of the culture media were collected, and IL-2 contents were assessed using an IL-2 assay kit (BD Biosciences Pharmingen, San Jose, CA).

For the assay for cell viability, cells were incubated under the above conditions for 12 h, and the percentage of cell viability was measured with light microscopy by observing trypan blue exclusion (usually ~200 cells were assessed).

Assay for IL-2 mRNA expression in Jurkat cells (semi-quantitative RT-PCR). Jurkat cells were pre-incubated in 90-mm culture dishes filled with 10 ml RPMI (at $2\text{–}4 \times 10^5$ cells/ml) in the presence or absence of FK506, $ZnSO_4$, $ZnCl_2$, $FeCl_2$, $FeCl_3$, $CaCl_2$, or $MgCl_2$ for several minutes (to assess the combinatorial effect of FK506 and Zn^{2+}, cells were pre-incubated in the presence or absence of FK506 and/or $ZnSO_4$ for 30 min). ConA or PMA (1 μM) and IM (0.5 μM) were then added to the media, and cells were further incubated for 3 h, collected by centrifugation (400*g*, 3–4 min), and lysed with 1 ml of ISOGEN (Wako Pure Chemical Industries, Japan). Total RNA was prepared from them, and 1 μg of total RNA was reversely transcribed into cDNA according to the manufacturer's instructions (for ISOGEN). PCR amplification (34 cycles) was performed as described [33,34] using the following primers: 5′-AT GTACAGGATGCAACTCCTGTCTT-3′ and 5′-GTT AGTGTTGAGATGATGCTTTGAC-3′ for IL-2 and 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCAC CACCCTGTTGCTGTA-3′ for glyceraldehyde 3-phosphate dehydrogenase (G3PDH). PCR products were then separated by 1% agarose gel electrophoresis and visualized with ethidium bromide. The visualized bands were digitized and quantified using Adobe Photoshop and NIH Image software.

Statistics. Statistical significance was assessed by ANOVA (post hoc Fisher's protected least significant difference).

Results

Effect of Zn^{2+} on ConA-induced IL-2 production in Jurkat cells

IL-2 production can be induced by stimulation with the mitogen, ConA, via the T-cell receptor (TCR) and subsequent activation of both CN and mitogen-activated protein kinase (MAPK) pathways in Jurkat cells. Since Zn^{2+} inhibits CN activity in vitro [32], we tried to determine whether exogenously added Zn^{2+} can inhibit IL-2 production in Jurkat cells (Fig. 1A). As expected, Jurkat cells did not produce IL-2, whereas ConA stimulated IL-2 production, which was greatly suppressed by the addition of 0.2 mM Zn^{2+} or 1 μM FK506 (an inhibitor of calcineurin). Importantly, the addition of 0.2 mM Zn^{2+} did not significantly affect cell viability (Fig. 1B), indicating that the inhibitory effect of Zn^{2+} on IL-2 production would not be exerted via cytotoxicity.

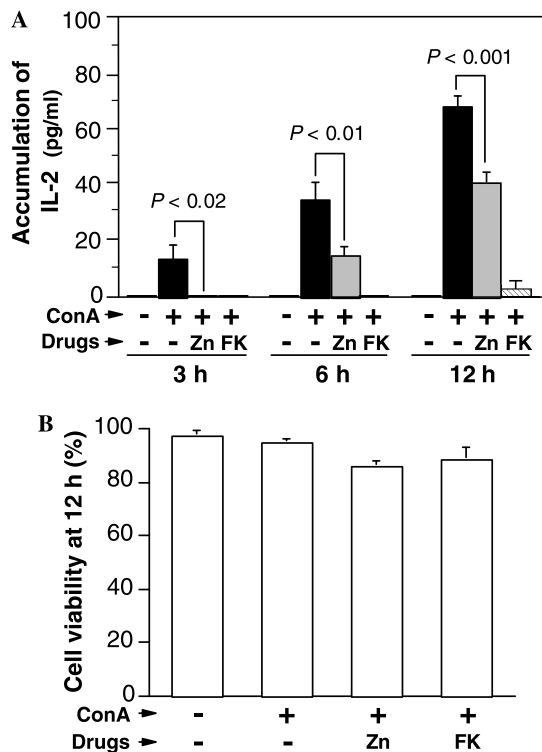

Fig. 1. Effects of Zn^{2+} on IL-2 production and cell viability in Jurkat cells. (A) Cells were incubated for 3, 6, and 12 h in the presence or absence of ConA (25 μg/ml), $ZnSO_4$ (0.2 mM), and/or FK506 (1 μM), and IL-2 contents in the culture media were assessed as described under Materials and methods. (B) Cells were incubated for 12 h in the presence or absence of ConA (25 μg/ml) and $ZnSO_4$ (0.2 mM), and cell viability was assessed as described under Materials and methods. Note that $ZnSO_4$ and FK506 were added several minutes before the addition of ConA.

We then examined the effect of Zn^{2+} on IL-2 mRNA expression in Jurkat cells (Figs. 2B and C). Zn^{2+} at 0.03–0.3 mM inhibited IL-2 expression in a dose-dependent manner (Fig. 2B), while Fe^{2+}, Fe^{3+}, Ca^{2+}, or Mg^{2+} at 0.3 mM did not inhibit IL-2 expression (Fig. 2C), although both Zn^{2+} and Fe^{2+} are elements of the catalytic domain of CN [15–18]. In the absence of ConA, most of the cells floated in the medium (Fig. 2D-a), but, in its presence, cells started to attach to the substratum (Fig. 2D-b). Again, the addition of 0.1–0.3 mM Zn^{2+} did not affect the cell morphology, and the cells appeared to be healthy (Fig. 2D-c,d). Nevertheless, these concentrations of Zn^{2+} thoroughly suppressed ConA-induced IL-2 production (Figs. 2B and C), suggesting that this suppressive effect of Zn^{2+} on IL-2 production is not exerted via the cytotoxicity of Zn^{2+}.

Effect of Zn^{2+} on PMA/ionomycin-stimulated IL-2 production

PMA (PKC activator) and ionomycin (IM: calcium ionophore) can stimulate IL-2 production by mimicking the signals via TCR in T-cells (Fig. 5). We examined the

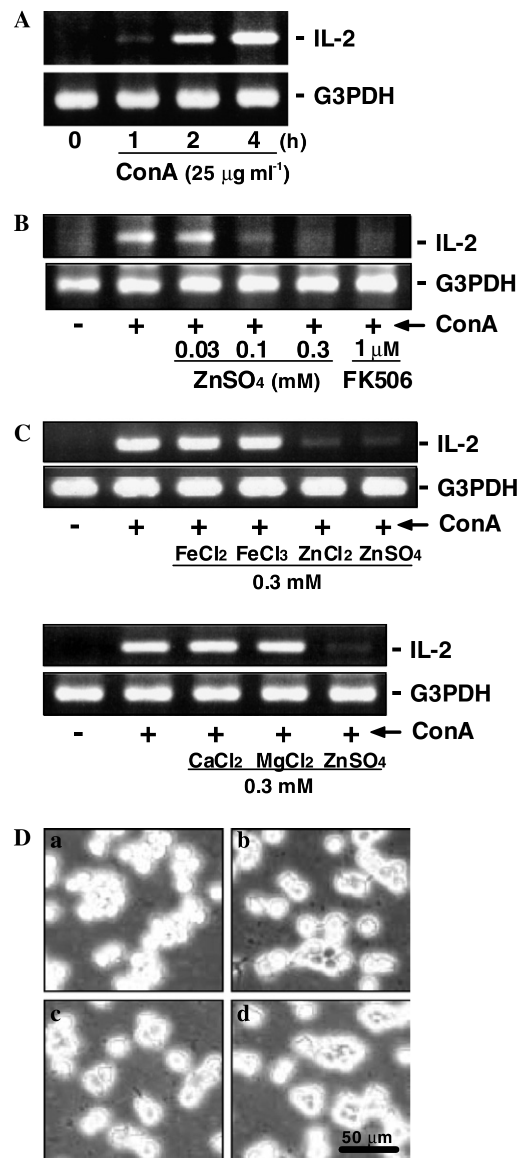

Fig. 2. Effects of Zn^{2+} and other metal ions on ConA-induced IL-2 mRNA expression in Jurkat cells. (A) Cells were incubated for the indicated periods in the presence of ConA (25 μg/ml). RNAs were prepared from the cells, and RT-PCR was performed to detect IL-2 and G3PDH mRNAs. (B) Cells were pre-incubated with 0–0.3 mM $ZnSO_4$ or 1 μM FK506 for several minutes and, after addition of ConA (25 μg/ml), they were further incubated for 3 h. RNAs were prepared from the cells, and RT-PCR was performed for IL-2 and G3PDH expression. Zn^{2+} suppressed ConA-induced IL-2 expression in a dose-dependent manner. (C) Cells were pre-incubated with the indicated metal ions (0.3 mM) for several minutes, and, after addition of ConA (25 μg/ml), they were further incubated for 3 h. RNAs were prepared from the cells, and RT-PCR was performed for IL-2 and G3PDH expression. ConA-induced IL-2 expression was suppressed by $ZnSO_4$ or $ZnCl_2$ but not by the other metal ions. (D) Cells were incubated for 3 h in the absence (a) or presence of ConA (25 μg/ml) (b), 0.1 mM $ZnSO_4$ plus ConA (c), or 0.3 mM $ZnSO_4$ plus ConA (d) (note that $ZnSO_4$ was added several minutes before the addition of ConA), and the cell morphology was observed under a phase-contrast microscope. In the absence of ConA, loose cell aggregates floated in the medium (a), but cells adhered to the bottom of the dishes in the presence of ConA (b–d). The cell morphology was not affected by the addition of 0.1–0.3 mM $ZnSO_4$ (c,d).

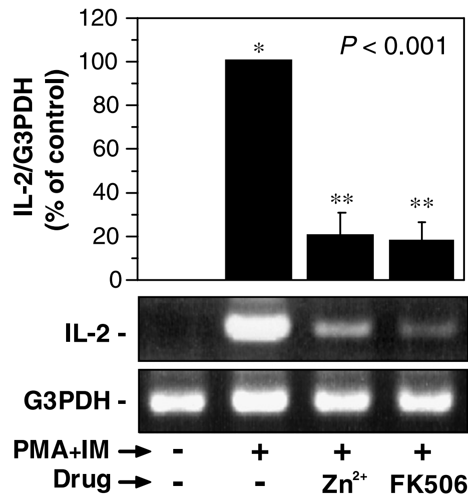

Fig. 3. Effect of Zn^{2+} on PMA/IM-induced IL-2 mRNA expression in Jurkat cells. Cells were pre-incubated with or without 0.3 mM $ZnSO_4$ or 0.3 μM FK506 for several minutes, and, after addition of PMA (1 μM) and ionomycin (IM: 0.5 μM), they were further incubated for 3 h (note that all the cultures contain 0.1% EtOH and 0.15% DMSO as vehicles). RNAs were prepared from the cells, and RT-PCR was performed for IL-2 and G3PDH expression. The bands were quantified, and normalized signals (IL-2/G3PDH) were converted to a percent of the control. The mean ± SD values of three independent experiments are shown in the graph above a representative image of RT-PCR. $^{**}P < 0.001$ versus *control.

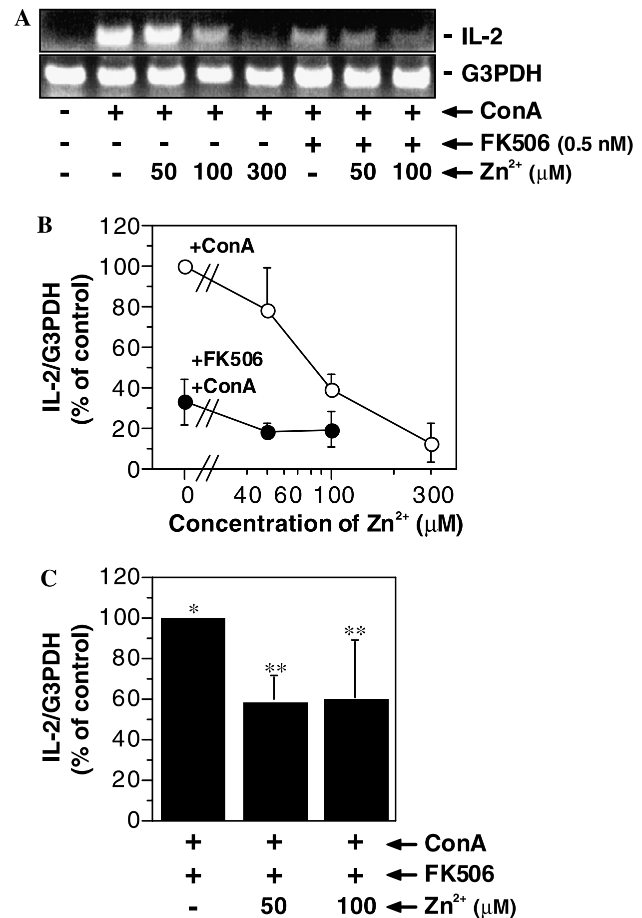

Fig. 4. Combined effects of Zn^{2+} and FK506 on ConA-induced IL-2 mRNA expression in Jurkat cells. (A) Cells were pre-incubated with 0–300 μM $ZnSO_4$ and/or 0.5 nM FK506 for 30 min, and, after addition of ConA (25 μg/ml), they were further incubated for 3 h. RNAs were prepared from the cells, and RT-PCR was performed for IL-2 and G3PDH expression. A representative image out of three independent experiments is shown. (B) The bands for IL-2 and G3PDH in (A) were quantified, and normalized signals (IL-2/G3PDH) were converted to a percent of the control (=IL-2/G3PDH induced by ConA). The mean ± SD values of three independent experiments are shown. (C) The bands for IL-2 and G3PDH in (A) were quantified, and normalized signals (IL-2/G3PDH) were converted to a percent of the control (=IL-2/G3PDH induced by ConA in the presence of FK506). The mean ± SD values of three independent experiments are shown. FK506 at 0.5 nM reduced the ConA-induced IL-2 production, which was further reduced by the addition of 50–100 μM Zn^{2+} in combination with FK506. $^{**}P < 0.03$ versus *control.

effect of Zn^{2+} on PMA/IM-stimulated IL-2 mRNA expression in Jurkat cells and found that Zn^{2+} suppressed IL-2 expression under the conditions as well (Fig. 3). The result indicates that the addition of Zn^{2+} results in inhibition somewhere downstream of the Ca^{2+} increase and/or PKC activation and suggests that exogenously added Zn^{2+} might increase $[Zn^{2+}]_i$ and inhibit intracellular CN, thereby suppressing IL-2 production (Fig. 5).

Combined effects of Zn^{2+} and FK506 on ConA-stimulated IL-2 production in Jurkat cells

We then examined the effects of Zn^{2+} and FK506 on IL-2 mRNA expression in Jurkat cells (Fig. 4). FK506 is a well-known immunosuppressive drug that inhibits CN activity via the immunophilin, FKBP12 [23–25]. FK506 at 0.5 nM suppressed IL-2 expression; however, in the presence of both FK506 and 50–100 μM Zn^{2+}, IL-2 expression was suppressed more strongly (Fig. 4). These results suggest that FK506 and Zn^{2+} may act on the same pathway involving CN.

Discussion

Role of Zn^{2+} in the regulation of CN activity in vivo

Zn^{2+} is essential for many cellular functions [1–8], and CN, which contains a Fe^{2+}–Zn^{2+} binuclear center in its catalytic domain [15–18], plays pivotal roles in many cellular functions in a variety of cells, e.g., in the regulation of immune systems and nerve systems [11–14,19–22]. Recently, we have shown that Zn^{2+} inhibits CN activity in vitro [32], but Fe^{2+} does not (our unpublished observation). It is thus possible that Zn^{2+} is not only an essential element for CN activity but also a negative regulator of CN activity, for instance, in T-cells, and, thus, of the immune system in vivo.

IL-2 production in T-cells is an index of immune power, and ConA is thought to stimulate IL-2 production

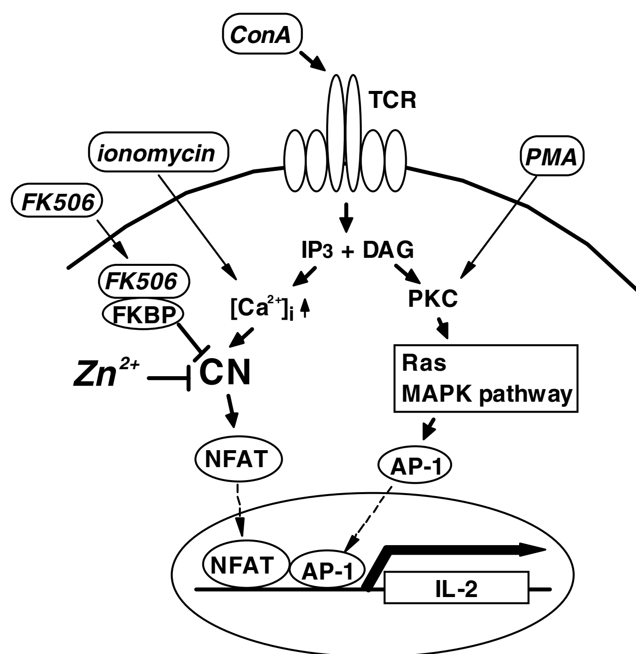

Fig. 5. Proposed scheme for the action of Zn^{2+} in T-lymphocytes. The mitogen, ConA, is thought to stimulate IL-2 production via a T-cell receptor (TCR) and subsequent activation of both MAPK/AP-1 and CN/NFAT pathways in T-lymphocytes, as described. The immunosuppressive drug, FK506, has been shown to inhibit CN via the FK506-binding protein 12 (FKBP). Cells control CN activity, in part, by regulating $[Zn^{2+}]_i$, while exogenously added Zn^{2+} appears to disturb (raise) $[Zn^{2+}]_i$ and inhibit CN activity, thereby suppressing IL-2 production. DAG; diacylglycerol. CN; calcineurin. NFAT; nuclear factor of activated T-cell. MAPK; mitogen-activated protein kinase. AP-1; a transcription factor consisting of either c-Jun homodimer or c-Fos/c-Jun heterodimer. IP_3; inositol 1,4,5-trisphosphate.

via the MAPK and CN pathways, as described in Fig. 5. In the present study, we have investigated the effect of exogenous Zn^{2+} on the ConA-induced IL-2 production in Jurkat T-cells and found that Zn^{2+} indeed suppressed IL-2 production (Fig. 1A) and its mRNA expression (Fig. 2B) in an in vitro culture. Because Zn^{2+} is known to block Ca^{2+} channels [35], it seemed possible that Zn^{2+} might suppress ConA-induced IL-2 expression by blocking a ConA-stimulated Ca^{2+} increase rather than inhibiting CN activity. However, Zn^{2+} suppressed IM/PMA-stimulated IL-2 expression (Fig. 3), indicating that the action point of Zn^{2+} should reside at least in the downstream of the Ca^{2+} increase in the cells (Fig. 5). Furthermore, since most of the Zn^{2+}-treated cells were viable (Fig. 1B) and looked healthy (Fig. 2D) and some other metal ions tested did not affect the IL-2 expression (Fig. 2C), the inhibitory effect of Zn^{2+} on IL-2 production should not be due to broad cytotoxicity of the ion but to a somewhat specific one, possibly exerted via the inhibition of CN activity (Fig. 5).

To speculate whether the inhibition of CN is one of the in vivo roles of Zn^{2+}, it is important to note that the effective concentrations of Zn^{2+} (10 nM–10 μM) for CN inhibition in vitro [32] are below the physiological concentrations of the ion (e.g., approximately 10–15 μM in adult human plasma) [36,37]. Here we have shown that exogenously added Zn^{2+} suppresses IL-2 production in an in vitro cell culture (Figs. 1–4), possibly by disturbing $[Zn^{2+}]_i$. Taken together, these findings indicate that cells may control CN activity in vivo by regulating $[Zn^{2+}]_i$.

Zn^{2+} as an immunosuppressive drug

Since the immunosuppressive drugs cyclosporin A and FK506 were found to inhibit CN via immunophilins [23–25], CN has been recognized as a therapeutic target for some diseases involving the immune system and treatment after transplantation. While the drugs have been utilized medically, they exhibit adverse effects, such as renal damage and hyperglycemia [38,39]. Thus, improvement of the known drugs (drug design) and the development/discovery of novel immunosuppressive reagents have been expected.

Our findings that Zn^{2+} inhibits the activity of a purified CN in vitro [32] and suppresses IL-2 production in an in vitro culture of Jurkat cells (this study) raise the possibility that Zn^{2+} may be utilized as an immunosuppressive and/or anti-inflammatory drug in vivo. Since ionic conditions in our body must be well regulated, exogenously added Zn^{2+} may not only suppress intracellular CN but also disturb some other cellular functions in vivo, which results in adverse effects. However, because relatively low concentrations of Zn^{2+} and FK506 exhibited an additive inhibitory effect on IL-2 production in Jurkat cells (Fig. 4), Zn^{2+} may be utilized at least as a supplemental drug to reduce the dose of FK506 and, thus, its adverse effects. It is thus possible that the general ion of Zn^{2+} may have therapeutic potential in the treatment of T-cell related inflammation, such as ulcerative colitis and Crohn's disease, especially in patients requiring immunosuppressive agents [40].

Acknowledgments

This work was supported in part by grants (Y.K. and K.H.) from the Ministry of Education, Science, Sports and Culture of Japan.

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