*Short Communication***Modulation of the Substance P Release From Cultured Rat Primary Afferent Neurons by Zinc Ions**He-Bin Tang^{1,2}, Kanako Miyano², and Yoshihiro Nakata^{2,*}¹College of Pharmacy, South-Central University for Nationalities, No. 708, Minyuan Road, Wuhan 430074, PR China²Department of Pharmacology, Graduate School of Biomedical Sciences, Hiroshima University, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8553, Japan

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Abstract. The present study was conducted to determine whether zinc ions are involved in the modulation of substance P release from cultured rat dorsal root ganglion (DRG) neurons. We show here that that lower concentrations (10^{-8} – 10^{-7} M) of zinc ions may evoke an extracellular Ca^{2+} influx through L-, N-, and T-type voltage-dependent Ca^{2+} channels, thereby increasing the substance P release from cultured DRG neurons, but higher concentrations (10^{-6} – 10^{-4} M) of zinc ions attenuate or even completely mask these responses. Zinc ions therefore seem to be an important modulator of substance P release from primary afferent neurons.

Keywords: dorsal root ganglion (DRG), substance P release, zinc ion

Zinc ion, an important divalent metal cation that acts as a novel intracellular second messenger because it plays numerous diverse regulatory roles in maintaining and regulating cellular functions in almost every cell in the body (1). For example, zinc ions regulate the conductivity of voltage-dependent Ca^{2+} channels (VDCCs) and thereby affect intracellular Ca^{2+} -signaling transduction (2). The release of zinc ions into the extracellular fluid of the spinal cord is involved in the modulation of nociceptive responses (3). Moreover, the lowering of vesicular zinc ions in the dorsal horn of the spinal cord may also reduce pain thresholds and cause neuropathic pain in mice (4). However, other functional evidence has indicated that substance P (a well-known tachykinin peptide) functions as an important neurotransmitter and/or as a primary afferent modulator, thereby potentiating excitatory input to nociceptive neurons in the peripheral and central nervous systems (5, 6). The cellular signal transduction by the influx of the extracellular Ca^{2+} and out of the intracellular Ca^{2+} stores in the rapid or slow release of substance P (7) from cultured DRG neurons suggested the possibility that zinc ions are involved in the substance P release from primary afferent neurons through the regulation of the Ca^{2+} -

sensitive signaling transduction.

The purpose of this investigation was therefore to ascertain whether a change in the extracellular zinc ion concentration and/or the chelation of intracellular zinc ions may influence the substance P release from cultured DRG neurons using a highly sensitive radioimmunoassay and a $^{45}\text{Ca}^{2+}$ -uptake assay.

The DRGs of 6-week-old male Wistar rats were dissociated into single cells (6 DRGs/dish or 3 DRGs/well) according to a previously described method (8). The cells were cultured in 35-mm dishes or 24-well plates in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical, Tokyo) plus 10% heat-inactivated horse serum, 1% penicillin/streptomycin, and 200 mM glutamine and maintained at 37°C in a water-saturated atmosphere with 5% CO_2 for 5 days before the experiment. All animal procedures were performed in accordance with the Guide for Animal Experimentation, Hiroshima University and the Committee of Research Facilities for Laboratory Animal Sciences, Graduate School of Biomedical Sciences, Hiroshima University, Japan.

The data are presented as the mean $\pm$ S.E.M. from at least three independent experiments. Statistical analyses were performed using a one-way analysis of variance (ANOVA) with Bonferroni post-test correction for multiple comparisons. All statistical analyses were undertaken using the GraphPad Prism statistical package

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(GraphPad Software, La Jolla, CA, USA). Significance was set at a value of $P < 0.05$ (two-tailed).

The observations obtained from the measurement of substance P release using a radioimmunoassay (6) indicated that a 30-min exposure of DRG neurons to zinc chloride (ZnCl_2 ; Sigma Chemical, St. Louis, MO, USA) at a concentration range of 10^{-8} – 10^{-4} M in Krebs–HEPES buffer (110 mM NaCl, 4.5 mM KCl, 2 mM CaCl_2 , 1.2 mM MgSO_4 , 1.2 mM KH_2PO_4 , 25 mM NaHCO_3 , 11.7 mM D-glucose, 5 mM HEPES) resulted in a bell-shaped curve for the substance P release (Fig. 1A). Lower concentrations (10^{-8} – 10^{-7} M) of ZnCl_2 triggered a significant dose-dependent increase in the release of substance P from DRG neurons, whereas it caused a gradual decrease in the substance P release at the higher concentrations (10^{-6} – 10^{-4} M). A separate study examined the effect of a single application of the zinc ion chelator *N,N,N',N'*-tetrakis(2-pyridylmethyl) ethylenediamine (9) (TPEN, Sigma) at a concentration range of 10^{-7} – 10^{-5} M on the spontaneous release of substance P from cultured DRG neurons and observed a maximum inhibitory effect induced by TPEN at 10^{-6} M (unpublished data). Figure 1A shows the ZnCl_2 -induced substance P release was completely inhibited by the pretreatment with TPEN ($1 \mu\text{M}$). In contrast to the presence of extracellular Ca^{2+} , only at a high concentration of $100 \mu\text{M}$, ZnCl_2 exhibited a weak tendency to increase substance P release level in the absence of extracellular Ca^{2+} (Fig. 1B), thus suggesting that the ZnCl_2 -induced substance P release might be mainly due to the influx of extracellular Ca^{2+} . In addition, a zinc-sensing receptor triggers the Ca^{2+} release from intracellular Ca^{2+} stores (10); therefore, the possibility cannot be ruled out that in the absence of extracellular Ca^{2+} , the addition of a high concentration of zinc ions induces the Ca^{2+} release from intracellular Ca^{2+} stores, thereby leading to only a slight increase in the substance P release. Based on the data shown in Fig. 1, A and B, incubation with 10^{-7} M ZnCl_2 was selected as an appropriate experimental condition to further examine the involvement of the VDCCs in the ZnCl_2 -induced substance P release from cultured DRG neurons. As shown in Fig. 1C, the pharmacological blockade of any of the L-, N-, and T-type VDCCs by pretreatment with the respective inhibitor (nicardipine, ω -conotoxin-GVIA, and amiloride) significantly attenuated the increase in the ZnCl_2 -induced substance P release. However, a single application of any of these three Ca^{2+} -channel inhibitors did not have any significant effect on the level of spontaneous substance P release (Fig. 1C). The doses of these three Ca^{2+} channel inhibitors should also be appropriate for the respective response inhibition according to the distinct pharmacological properties of

Fig. 1. Effect of ZnCl_2 on the substance P release from cultured DRG neurons. Some cells were left untreated as a control, and all other cells were pretreated with either TPEN ($1 \mu\text{M}$) for 30 min or one of the three VDCC inhibitors ($5 \mu\text{M}$ nicardipine, $1 \mu\text{M}$ ω -conotoxin-GVIA, and $100 \mu\text{M}$ amiloride) for 15 min at 37°C . The pretreated cells were washed and then both the pretreated cells and control cells were continually stimulated by various concentrations of ZnCl_2 (A and B) or $0.1 \mu\text{M}$ of ZnCl_2 (C) in Krebs–HEPES buffer with peptidase inhibitors ($1 \mu\text{M}$ phosphoramidon, $4 \mu\text{g/ml}$ bacitracin, and $1 \mu\text{M}$ captopril; Sigma) or peptidase inhibitors only in Ca^{2+} -containing (A and C) or Ca^{2+} -free (B) Krebs–HEPES buffer for 30 min at 37°C as a control. The data are obtained from 3 (A), 3 (B), or 4–6 (C) separate experiments. C: The numbers in parentheses indicate the numbers of experiments. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$: Bonferroni post-test correction for multiple comparisons, two-tailed; A: 9 comparisons in 10 groups, B: 4 comparisons in 5 groups and C: 4 comparisons in 5 groups.

these inhibitors reported in several previous observations (11, 12). In addition, the influence of the chloride ions from the exogenous addition of ZnCl_2 (10^{-8} – 10^{-4} M) may be ignored in the present study because the total chloride ion levels were very high (approximately 120 mM) in the assay buffers (Ca^{2+} -containing and Ca^{2+} -free Krebs–HEPES buffer). Together with the results shown in Fig. 1, these findings indicate that zinc ions seem to be involved in the substance P release through an extracellular Ca^{2+} -dependent signaling cascade. This is the first report to describe zinc ions modulating the substance P release from primary afferent neurons.

Next, the possible effect of zinc ions on the extracellular Ca^{2+} influx was further examined using a $^{45}\text{Ca}^{2+}$ (MP Biomedicals, Irvine, CA, USA) uptake assay (13). A 10-min exposure of cultured DRG cells to ZnCl_2 at a concentration range of 10^{-8} – 10^{-4} M resulted in a bell-shaped curve for the $^{45}\text{Ca}^{2+}$ uptake, and the ZnCl_2 -induced extracellular Ca^{2+} influx was almost completely abolished by pretreatment with 1 μM TPEN (Fig. 2A). As expected, the extracellular Ca^{2+} influx induced by 1 μM ZnCl_2 was significantly attenuated by the pretreatment with any of the L-, N-, and T-type VDCC inhibitors, 5 μM nicardipine, 1 μM ω -conotoxin-GVIA, and 100 μM amiloride (Fig. 2B). These results indicated that lower concentrations of exogenous zinc ions may evoke the extracellular Ca^{2+} influx through the VDCCs, but attenuate the extracellular Ca^{2+} influx at the higher concentrations. In addition, an observation by Magistretti et al. (14) indicated that zinc ions at higher concentrations (30 μM) may compete with Ca^{2+} for a binding site within Ca^{2+} channels (including the L- and N-type VDCCs) and thereby produce its blocking effect in rat palaeocortical neurons. This observation may help us to partially understand the dual effect of zinc ions on the extracellular Ca^{2+} influx through multiple types of VDCCs. Regrettably, the precise mechanisms underlying such zinc ion regulation of the extracellular Ca^{2+} influx remain to be fully elucidated. Therefore, the data obtained from the $^{45}\text{Ca}^{2+}$ -uptake measurement further affirmed the involvement of extracellular Ca^{2+} influx through the L-, N-, and T-type VDCCs in the ZnCl_2 -induced substance P release. However, it is necessary to further examine the effects of changing the intracellular concentration of either the zinc ions and/or other heavy metal ions on the release of substance P in future studies because TPEN has a very high affinity for divalent heavy metal cations, including zinc, iron, and manganese ions, and much smaller affinity for Ca^{2+} and magnesium ions (9). In addition, recent research has indicated that lower concentrations of zinc ions activated the transient receptor potential ankyrin 1 (TRPA1), while at concentrations higher than 300 μM , zinc ions

Fig. 2. Effect of ZnCl_2 on the $^{45}\text{Ca}^{2+}$ uptake into cultured DRG cells. Some cells were left untreated as a control; the other cells were pretreated with 1 μM TPEN (A: 12 experimental groups) for 30 min or one of the three VDCC inhibitors (nicardipine, ω -conotoxin-GVIA, and amiloride) (B: 5 experimental groups) for 15 min at 37°C and then all cells were stimulated with or without ZnCl_2 or ZnCl_2 plus TPEN for 10 min at 37°C in $^{45}\text{Ca}^{2+}$ -labeled buffer (final concentration of 2 $\mu\text{Ci}/\text{ml}$ in Krebs–HEPES buffer without 2 mM CaCl_2). After extensive washing in Ca^{2+} -containing buffer, the radioactivity of $^{45}\text{Ca}^{2+}$ accumulated in the cells in each well was counted with an Aloka LSC-5100 liquid scintillation counter (Aloka, Tokyo). The numbers in parentheses indicate the numbers of experiments. The data are obtained from 3 separate experiments. * $P < 0.05$ and ** $P < 0.01$; Bonferroni post-test correction for multiple comparisons, two-tailed; A: 11 comparisons in 12 groups, B: 4 comparisons in 5 groups.

also exhibited an inhibitory effect on TRPA1 (15). It is well known that TRPA1 is a nonselective cation channel expressed in DRG neurons and activated by a number of irritants such as mustard oil (allyl isothiocyanate), cinnamon oil (cinnamaldehyde), and garlic (allicin) (16). We therefore next examined the possibility of TRPA1 involvement in the zinc ion-induced substance P release in the present study and observed an inhibitory effect [$132 \pm 36\%$ of the control ($100 \pm 13\%$, $n = 3$), $n = 3$; $P > 0.05$ when compared to ZnCl_2 alone] of 20 μM ruthenium red (a non-selective TRP channel blocker, Sigma) on the substance P release induced by 10^{-7} M

ZnCl₂ ($208 \pm 12\%$ of the control, $n = 3$; $P < 0.05$ when compared to the control). In addition, ruthenium red used alone ($78 \pm 23\%$ of the control, $n = 3$; $P > 0.05$ when compared to the control) did not significantly influence the spontaneous release of substance P from cultured DRG neurons. Based on the present data, the TRPA1 involvement in the zinc ion-induced substance P release could therefore not be ruled out.

In conclusion, the present study demonstrated that lower concentrations of zinc ions may evoke an extracellular Ca²⁺ influx through the L-, N-, and T-type VDCCs, thereby increasing the release of substance P from cultured DRG neurons, but at higher concentrations, zinc ions seem to block the activation of these voltage-dependent calcium channels, thereby leading to an inhibition of the extracellular Ca²⁺ influx and attenuation of the release of substance P. These data suggest that zinc ions seem to serve as an important modulator of substance P release in the control of nociceptive information transmission.

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References

- 1 Yamasaki S, Sakata-Sogawa K, Hasegawa A, Suzuki T, Kabu K, Sato E, et al. Zinc is a novel intracellular second messenger. *J Cell Biol*. 2007;177:637–645.
- 2 Sun HS, Hui K, Lee DW, Feng ZP. Zn²⁺ sensitivity of high- and low-voltage activated calcium channels. *Biophys J*. 2007;93:1175–1183.
- 3 Larson AA, Kitto KF. Manipulations of zinc in the spinal cord, by intrathecal injection of zinc chloride, disodium-calcium-EDTA, or dipicolinic acid, alter nociceptive activity in mice. *J Pharmacol Exp Ther*. 1997;282:1319–1325.
- 4 Jo SM, Danscher G, Schröder HD, Suh SW. Depletion of vesicular zinc in dorsal horn of spinal cord causes increased neuropathic pain in mice. *Biomaterials*. 2008;21:151–158.
- 5 Cao YQ, Mantyh PW, Carlson EJ, Gillespie AM, Epstein CJ, Basbaum AI. Primary afferent tachykinins are required to experience moderate to intense pain. *Nature*. 1998;392:390–394.
- 6 Tang HB, Li YS, Arihiro K, Nakata Y. Activation of the neurokinin-1 receptor by substance P triggers the release of substance P from cultured adult rat dorsal root ganglion neurons. *Mol Pain*. 2007;3:42.
- 7 Tang HB, Nakata Y. The activation of transient receptor potential vanilloid receptor subtype 1 by capsaicin without extracellular Ca²⁺ is involved in the mechanism of distinct substance P release in cultured rat dorsal root ganglion neurons. *Naunyn Schmiedeberg's Arch Pharmacol*. 2008;377:325–332.
- 8 Tang HB, Shiba E, Li YS, Morioka N, Zheng TX, Ogata N, et al. Involvement of voltage-gated sodium channel Na_v1.8 in the regulation of the release and synthesis of substance P in adult mouse dorsal root ganglion neurons. *J Pharmacol Sci*. 2008;108:190–197.
- 9 Arslan P, Di Virgilio F, Beltrame M, Tsien RY, Pozzan T. Cytosolic Ca²⁺ homeostasis in Ehrlich and Yoshida carcinomas. A new, membrane-permeant chelator of heavy metals reveals that these ascites tumor cell lines have normal cytosolic free Ca²⁺. *J Biol Chem*. 1985;260:2719–2727.
- 10 Hershfinkel M, Moran A, Grossman N, Sekler I. A zinc-sensing receptor triggers the release of intracellular Ca²⁺ and regulates ion transport. *Proc Natl Acad Sci U S A*. 2001;98:11749–11754.
- 11 Tang CM, Presser F, Morad M. Amiloride selectively blocks the low threshold (T) calcium channel. *Science*. 1988;240:213–215.
- 12 Andoh T, Kuraishi Y. Direct action of immunoglobulin G on primary sensory neurons through Fc gamma receptor I. *FASEB J*. 2004;18:182–184.
- 13 Tang HB, Inoue A, Oshita K, Nakata Y. Sensitization of vanilloid receptor 1 induced by bradykinin via the activation of second messenger signaling cascades in rat primary afferent neurons. *Eur J Pharmacol*. 2004;498:37–43.
- 14 Magistretti J, Castelli L, Taglietti V, Tanzi F. Dual effect of Zn²⁺ on multiple types of voltage-dependent Ca²⁺ currents in rat palaeocortical neurons. *Neuroscience*. 2003;117:249–264.
- 15 Hu H, Bandell M, Petrus MJ, Zhu MX, Patapoutian A. Zinc activates damage-sensing TRPA1 ion channels. *Nat Chem Biol*. 2009;5:183–190.
- 16 Jordt SE, Bautista DM, Chuang HH, McKemy DD, Zygmunt PM, Högestätt ED, et al. Mustard oils and cannabinoids excite sensory nerve fibres through the TRP channel ANKTM1. *Nature*. 2004;427:260–265.