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Original Article

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Modulation of Dopaminergic Neuronal Excitability by Zinc through the Regulation of Calcium-related Channels

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Depending on the intracellular buffering of calcium by chelation, zinc has the following two apparent effects on neuronal excitability: enhancement or reduction. Zinc increased tonic activity in the depolarized state when neurons were intracellularly dialyzed with EGTA but attenuated the neuronal activity when BAPTA was used as an intracellular calcium buffer. This suggests that neuronal excitability can be modulated by zinc, depending on the internal calcium buffering capacity. In this study, we elucidated the mechanisms of zinc-mediated alterations in neuronal excitability and determined the effect of calcium-related channels on zinc-mediated alterations in excitability. The zinc-induced augmentation of firing activity was mediated via the inhibition of small-conductance calcium-activated potassium (SK) channels with not only the contribution of voltage-gated L-type calcium channels (VGCCs) and ryanodine receptors (RyRs), but also through the activation of VGCCs via melastatin-like transient receptor potential channels. We suggest that zinc modulates the dopaminergic neuronal activity by regulating not only SK channels as calcium sensors, but also VGCCs or RyRs as calcium sources. Our results suggest that the cytosolic calcium-buffering capacity can tightly regulate zinc-induced neuronal firing patterns and that local calcium-signaling domains can determine the physiological and pathological state of synaptic activity in the dopaminergic system.

Key words: Calcium-activated non-selective cation, Dopamine system, Electrophysiology, Patch clamp recording, Spike frequency, Rat

INTRODUCTION

Zinc ion (Zn^{2+}) is the most abundant transition metal ion in biological organisms, with mammalian cells containing an estimated 100~500 μM of total Zn^{2+} [1, 2]. Zn^{2+} is highly enriched at many, but not all, glutamatergic nerve terminals. In the brain, Zn^{2+} is released upon neuronal activity and is crucial for the control of physiological and pathophysiological brain functions. Zn^{2+} enables the modulation of neuronal excitability in the substantia nigra pars compacta (SNc) in two ways—excitation or inhibition—by altering the gating properties of voltage-gated transient A-type K^+ channels [3]. Noh et al. proposed that Zn^{2+} increases the bursting activity of SNc neurons in a hyperpolarized state through the reduction of the transient outward K^+ current (I_A) and decreases its tonic activity in depolarized states when I_A is increased. However, this proposal does not support previous observations that in depolarized states, Zn^{2+} enhances the firing activity of dopaminergic neurons that are acutely isolated from the rat SNc [4]. These contradictory Zn^{2+}-mediated effects on excitability in SNc neurons at depolarized states were the result of investigations employing different intracellular Ca^{2+} chelators, namely, ethylene glycol tetraacetic acid (EGTA), and 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA). Therefore, it is important to determine whether the Zn^{2+}-elicited alteration of firing activity is dependent on the Ca^{2+}-buffering capacity of neurons, and on the cytosolic Ca^{2+}-dependent signaling processes.

In central nervous system neurons and many other cell types, transient elevations in intracellular Ca^{2+} trigger a wide variety

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of Ca^{2+}-dependent signaling events and processes including the regulation of firing activities and patterns. The Ca^{2+} signals are precisely restricted to local spatiotemporal domains, which are generated by a variety of different Ca^{2+} buffer systems that limit the diffusion of Ca^{2+} ions [5]. Ca^{2+} buffers—a class of cytosolic Ca^{2+}-binding proteins—act as modulators of short-lived intracellular Ca^{2+} signals. Each neuron is equipped with proteins such as Ca^{2+} channels, transporters, pumps, and Ca^{2+} buffers that together shape the intracellular Ca^{2+} signals. A recent study demonstrated that the absence of Ca^{2+}-binding proteins, such as parvalbumins, calbindins, and calretinins, leads to an increase in neuronal excitability through a reduction in cytosolic Ca^{2+}-buffering capacity, suggesting a link between the expression of cellular Ca^{2+}-binding proteins and excitability [6-8]. Alterations in the Ca^{2+}-calmodulin kinase systems have been associated with some strains of seizure-susceptible mice, suggesting that calmodulin-mediated processes may play a role in the development of altered neuronal excitability and in some forms of seizure disorders [9].

Bouts of action potentials can lead to an increase in the cytosolic Ca^{2+} concentration of neurons through the activation of voltage-gated Ca^{2+} channels (VGCCs) or Ca^{2+}-permeable transient receptor potential (TRP) channels, as well as through the release of Ca^{2+} from intracellular stores via Ca^{2+}-induced Ca^{2+} release mechanisms. As a result, increased cytosolic Ca^{2+} levels would control neuronal excitability by modulating many ion channels, including Ca^{2+}-activated K^+ (K_{Ca}) channels or Ca^{2+}-activated TRP channels, which regulate the firing activities and patterns of SNc dopamine neurons [10, 11]. In midbrain dopaminergic neurons, the steep Ca^{2+} concentration gradient enables it to cross the plasma membrane into neurons through open pores such as L-type VGCCs, which appear to play a major role in regulating firing activities and intracellular Ca^{2+} concentration dynamics [12, 13]. Ca^{2+} is also able to flow into the cytoplasm though the inositol trisphosphate receptor (IP_3R) and ryanodine receptor (RyR) ion channels in the ER membrane [14]. The influx of Ca^{2+} via VGCCs or IP_3R/RyR in a depolarized state leads to the activation of small-conductance K_{Ca} (SK) channels. This generates a large afterhyperpolarization that affects the interspike interval of pacemaker firing in the dopaminergic midbrain neurons [15]. It was recently reported that many types of TRP channels are widespread in midbrain dopamine neurons [16, 17] and that they play a critical role in Ca^{2+} homeostasis and the regulation of firing in dopaminergic SNc neurons [10].

In light of these recent reports, this study was undertaken to examine how Zn^{2+} contributes to altered neuronal excitability in dopaminergic SNc neurons, depending on the cytosolic Ca^{2+} capacity and Ca^{2+}-related channel processes, using a whole-cell patch recording in SNc neurons. We found that SK channel activity, which is influenced by changes in the cytosolic Ca^{2+} concentration under regulation by the Ca^{2+}-buffering capacity, was essential in the Zn^{2+}-mediated alteration of firing activity.

MATERIALS AND METHODS

Brain preparation

All experiments followed the guidelines issued by Ewha Womans University on the ethical use of animals for experimentation.

Slice preparation

The rats (Sprague-Dawley, age 11~20 days) were anesthetized with isoflurane and decapitation. The brain was quickly removed from the rat skull and immersed in ice-cold high-concentration sucrose solution containing (in mM): 201 sucrose, 3 KCl, 1.25 NaH_2PO_4, 2 $MgCl_2$, 2 $CaCl_2$, 26 $NaHCO_3$, and 10 $_D$-glucose. Coronal midbrain slice (300 μm) containing SN_C was cut in ice-cold high-concentration sucrose solution using a vibratome (VT1000S, Leica Microsystems, Germany). Before recording, slices were incubated at 30℃ for 60 min in artificial cerebrospinal fluid (ACSF: 95% O_2/5% CO_2) containing (in mM): 126 NaCl, 3 KCl, 1.25 NaH_2PO_4, 1.3 $MgSO_4$, 2.4 $CaCl_2$, 26 $NaHCO_3$, and 10 $_D$-glucose. After the recovery period, slices were maintained in ACSF at room temperature (20±3℃).

Dissociation of SNc neurons

Dissociated SNc dopamine neurons were prepared from rats (Sprague-Dawley, age 9~13 days) as described previously [4]. Whole brains from decapitated rats were quickly removed and placed in an ice-cold oxygenated N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES)-buffered saline containing (in mM): 135 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 25 $_D$-glucose, 10 HEPES (pH 7.3). Coronal slice (300~400 μm) containing SNc was cut using a vibratome (VT1000S, Leica). The dissected SNc segments were incubated in a fully oxygenated HEPES-buffered saline containing papain (10 U/ml; Worthington Biochemical Corp., Lakewood, NJ, USA) for 30 min at 36℃. Tissue segments were rinsed with enzyme-free saline and mildly triturated with a graded series of fire-polished Pasteur pipettes. Dissociated SN_C neurons were plated onto 35-mm Petri-dishes at room temperature (20±3℃).

Electrophysiological recordings

Whole-cell current clamp recordings for SNc slices

Whole-cell current clamp records were obtained from dopaminergic neurons in SNc slices as previously described [3]. Recordings were performed in a submersion-type chamber (2~4 ml/min

perfusion of oxygenated modified ACSF at 30~32℃) mounted on an upright microscope (Olympus BX51WI, Tokyo, Japan). Patch pipettes (3~4 MΩ) were pulled on a Narishige electrode puller PP-83 (Narishige, Tokyo, Japan) from Kimax borosilicate glass capillaries (KG-33, Precision Glass, Inc., Claremont, CA, USA) with an inner diameter of 1.2 mm and an outer diameter of 1.5 mm. The internal electrode solution contained (in mM): 115 K-gluconate, 10 HEPES, 1 $MgCl_2$, 10 KCl, 1 $CaCl_2$, 11 EGTA or 11 BAPTA, 4 Na_2-ATP (pH 7.3). Spikes in current-clamp mode were acquired with a multiclamp700B amplifier (Molecular Devices, Sunnyvale, CA, USA) and a Digidata-1440A A/D converter (Molecular Devices) using pClamp10 (Molecular Devices). Currents were Bessel-filtered at a cut-off frequency of 5 kHz and digitally sampled at 10 kHz. Data graphing and response analyses were performed with the GraphPad Prism8.0 (GraphPad Software Inc., La Jolla, CA, USA) and pClamp10 (Molecular Devices).

Whole-cell voltage-clamp recordings for dissociated SNc neurons

Whole-cell recordings were obtained from isolated SNc using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA) with a Digidata-1320A/D converter and analyzed using pClamp9 software (Molecular Devices). The Patch pipettes resistances were 3.0~3.5 MΩ. Recording pipettes were filled with (in mM): 135 CsCl, 2 $MgCl_2$, 10 HEPES, 11 EGTA, 4 ATP-Na_2 (pH 7.3). Series resistances were compensated (>50%) and monitored at regular intervals. Leakage and capacitive currents were subtracted on-line from active responses using P/5 protocols. Data graphing and response analyses were performed with the GraphPad Prism8.0 (GraphPad Software Inc.) and pClamp9 (Molecular Devices).

Single-channel recordings for dissociated SNc neurons

Pipettes were pulled from standard wall borosilicate glass capillaries (o.d., 1.5 mm; i.d.,1.2 mm; KG-33, Garner Glass) using a two-stage puller (PP-83, Narishige) and coated with sylgard (Dow Corning) or Sigmacote (Sigma) to reduce electrical noise. Pipette resistance was 10~20 MΩ. The data were filtered at 2 kHz (four-pole Bassel) and digitized at 20 kHz. Channel open frequency was analyzed by the pClamp10 using a 50% threshold detection criterion. Events briefer than 150 μs were ignored.

Solutions

For the current-clamp recordings of slices, a modified ACSF, in which NaH_2PO_4 was omitted and $MgSO_4$ was replaced with $MgCl_2$, was used as an extracellular solution to avoid any Zn^{2+}-related precipitates. For the measurement of voltage-gated Ca^{2+} channels in a voltage-clamp mode, the composition of bath solution in control was (in mM): 155 tetraethylammonium chloride, 10 $BaCl_2$, and 10 HEPES (pH 7.4). Bath solutions were added to the recording chamber, using a perfusion system that consisted of 5 polyethylene tubes arranged in parallel in one plane for rapid exchange. All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) and Tocris (Ellisville, MO, USA). For inside-out patch single-channel recordings of SK channels, bath and pipettes contained (mM): 140 KCl, 10 HEPES, 5 $MgCl_2$, pH 7.2. To activate SK channels, free calcium concentration in the bath solution was adjusted by addition of 100 μM $CaCl_2$.

Statistical analysis

Data are presented as mean±standard error mean (S.E.M). Statistical significance was estimated with two-tailed Student's *t*-tests for comparisons between two groups and was evaluated by using one-way repeated measures ANOVA followed by post hoc Tukey's multiple comparison test and Dunnett's post-hoc multiple comparison test for control.

RESULTS

Differential modulation of the excitability in SNc dopaminergic neurons by zinc, depending on internal calcium chelators

Dopaminergic SNc neurons recorded in juvenile rat brain slices were identified in a whole-cell patch-clamp by the presence of a prominent sag during hyperpolarizing current pulses, and repetitive firing due to anode break excitation, which are well-known criteria for identifying dopaminergic neurons in the SNc [4, 18, 19]. We had previously reported seemingly contradictory Zn^{2+}-mediated effects on the excitability of SNc neurons, where Zn^{2+} increased the excitability of dopaminergic neurons acutely isolated from the rat SNc [4], but decreased the firing activity in the dopaminergic neurons of SNc slices [3].

To investigate the influence of the Ca^{2+}-buffering capacity on Zn^{2+}-mediated changes in the excitability of SNc neurons, we examined the effects of Zn^{2+} on the activity patterns of SNc neurons that had received depolarizing pulses through EGTA- or BAPTA-containing intracellular solutions in a pipette. When the pipette contained EGTA, the evoked action potentials revealed regularity in the spike frequency patterns (Fig. 1A, Control). Zn^{2+} (100 μM) significantly increased the spike frequency to 181% (Fig. 1C, 100 μM Zn^{2+}), but this effect was not completely reversed after the washout (Fig. 1C, Wash). However, when using the same concentration of BAPTA, the pattern of firing activities evoked by depolarization showed the opposite effect, suggesting that the

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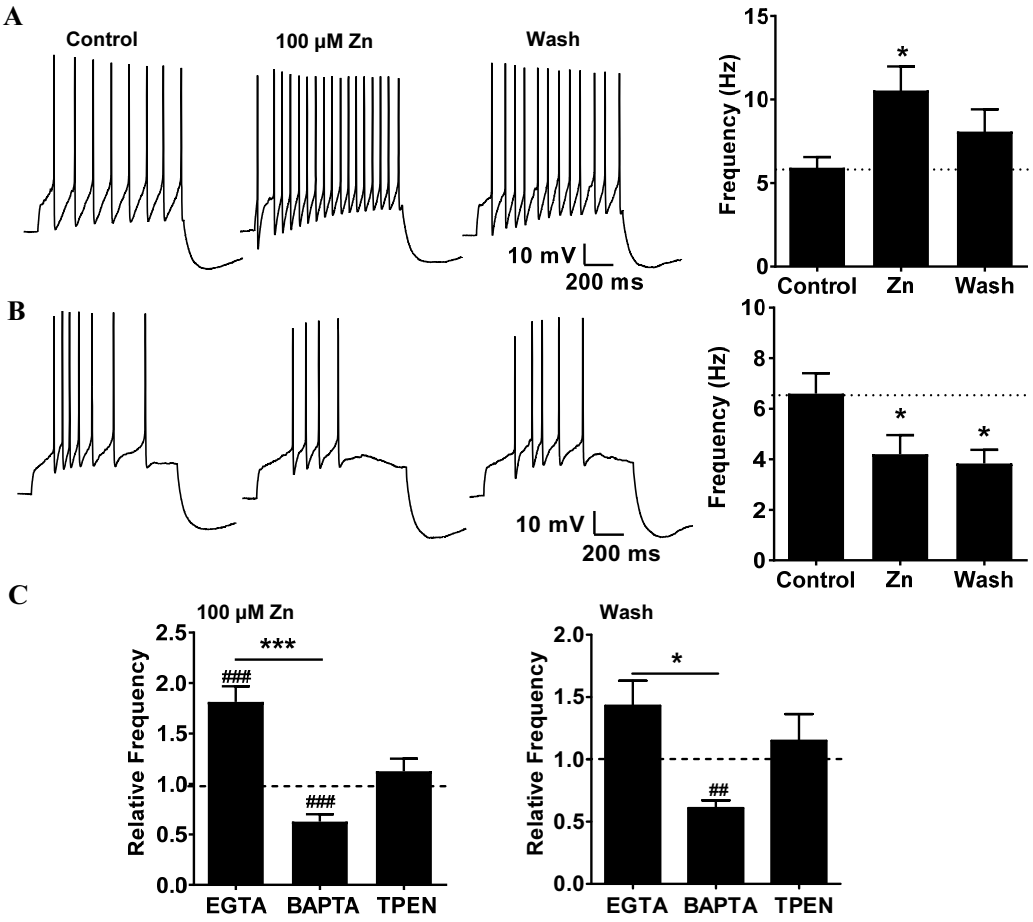

Fig. 1. Differential modulation of the excitability in SNc dopaminergic neurons by EGTA or BAPTA as internal Ca^{2+} chelators in the presence of Zn^{2+}. (A, B) *Left*, voltage responses of a SNc neuron to a depolarizing pulse (100 pA current injection for 1 s) in the absence (Control and Wash) and the presence of Zn^{2+} (100 μM Zn) were recorded using intracellular solutions with 11 mM EGTA (A) or 11 mM BAPTA (B) in the pipette solution. *Right*, effect of Zn^{2+} on the frequency of evoked action potentials. (A: Control, 5.92±0.64 Hz, n=13; Zn, 10.54±1.44 Hz, n=13; Wash, 8.08±1.33 Hz, n=12; p<0.05, one-way ANOVA; B: Control, 6.22±0.80 Hz, n=9; Zn, 4.11±0.87 Hz, n=9; Wash, 3.83±0.54 Hz, n=6; p<0.05, one-way ANOVA; *p<0.05, Dunnett's post-hoc multiple comparison test for control). (C) Summary of the relative frequency in the presence of Zn^{2+} (100 μM Zn) and after Zn^{2+} washout (Wash) in the EGTA-, BAPTA-, and TPEN-loaded condition (100 μM Zn: EGTA, 1.81±0.15, n=13; BAPTA, 0.63±0.07, n=9; TPEN, 1.13±0.13, n=4; p<0.001, one-way ANOVA; Wash: EGTA, 1.43±0.99, n=12; BAPTA, 0.61±0.06, n=6; TPEN, 1.15±0.21, n=4; p<0.05, one-way ANOVA; *p<0.05, ***p<0.001, Tukey's post-hoc multiple comparison test; ##p<0.01; ###p<0.001, one sample t-test, hypothetical value=1).

Ca^{2+}-buffering capacity might alter the Zn^{2+}-induced modulation of excitability. The appearance of spike-frequency adaptation patterns in the pipettes containing BAPTA (Fig. 1B) suggested that the spike-frequency adaptation in SNc dopaminergic neurons occurs due to the lowering of cytoplasmic Ca^{2+} levels, which may cause a decrease in the activity of SK channels [20, 21]. In experiments with BAPTA-containing pipettes, Zn^{2+} significantly decreased the spike frequency to 63% (Fig. 1C, 100 μM Zn^{2+}), but the firing activity did not recover completely after washout (Fig. 1C, Wash). BAPTA is also able to chelate Zn^{2+} influxes into the cytoplasm (K_D values: Ca^{2+}, 160×10^{-9} M; Zn^{2+}, 8×10^{-9} M)[22]. Therefore, we examined the effects of internal Zn^{2+} on spike frequencies using pipettes containing the Zn^{2+} chelator N,N,N',N'-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN, 50 μM), which has a very high affinity for Zn^{2+} ($K_D=2.6\times10^{-16}$ M), but relatively low affinities for Ca^{2+} ($K_D=4.0\times10^{-5}$ M) and Mg^{2+} ($K_D=2.0\times10^{-2}$ M) [23]. In experiments with TPEN-loaded pipettes, the Zn^{2+}-mediated increase in excitability was less pronounced than in the EGTA experiments (Fig. 1C), suggesting that the blocking of Zn^{2+}-mediated potentiation of spike activity may be due to the chelation of intracellular Zn^{2+} by both BAPTA and TPEN. However, we were unable to rule out the possibility that the membrane-permeable TPEN also chelates extracellular Zn^{2+}, and we could not explain why the presence of TPEN does not result in reduced activity via enhanced I_A. Therefore, we also tested the effect of extracellular Zn^{2+}-mediated augmentation of excitability on Ca^{2+} channels in the cell membrane

and the endoplasmic reticulum (ER).

Involvement of VGCCs and intracellular calcium stores in the zinc-mediated augmentation of excitability

In SNc neurons, bouts of action potential can increase the cytosolic Ca^{2+} concentration either through the activation of VGCCs, or through the release of Ca^{2+} from intracellular stores via Ca^{2+}-induced Ca^{2+} release signaling. The abrupt change in conductance enables Ca^{2+} to enter the neuronal cytoplasm rapidly through the open pores of L-type VGCCs in SNc dopaminergic neurons [24, 25]. First, we examined the changes in excitability in the presence of Cd^{2+} and Ni^{2+} to determine whether high-threshold VGCCs and low-threshold VGCCs are involved in the observed Zn^{2+}-induced increases in excitability. In the presence of Cd^{2+} (100 μM), the Zn^{2+}-induced increases in firing activities were completely blocked, and similar to the effect of BAPTA, this phenomenon did not recover after Zn^{2+} washout (Fig. 2A, *top*). In contrast, the presence of Ni^{2+} (100 μM) did not influence the Zn^{2+}-mediated in-

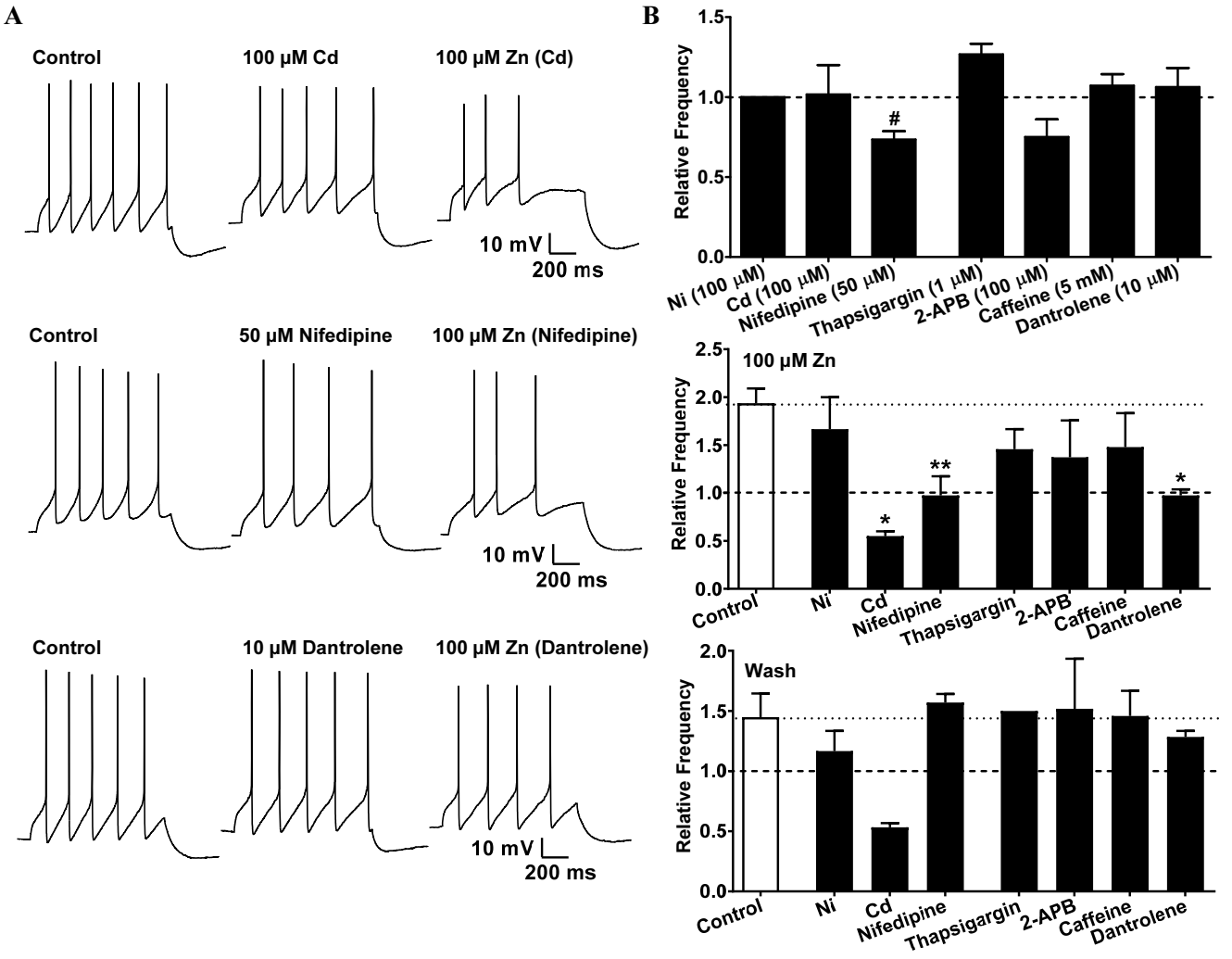

Fig. 2. Involvement of VGCCs and intracellular Ca^{2+} stores in the Zn^{2+}-mediated augmentation of excitability in SNc dopaminergic neurons. (A) Current-injected voltage raw traces by a depolarizing pulse (100 pA current injection for 1 s). *Top*, 100 μM Cd; *Middle*, 50 μM Nifedipine; *Bottom*, 10 μM Dantrolene. (B) *Top*, effects of the inhibition of VGCCs and ER-related factors on the firing activity frequency (100 μM Ni, 1.00±0.0, n=3; 100 μM Cd, 1.02±0.18, n=3; 50 μM Nifedipine, 0.74±0.05, n=8; 1 μM Thapsigargin, 1.27±0.07, n=4; 100 μM 2-APB, 0.75±0.11, n=6; 5 mM Caffeine, 1.07±0.07, n=4; 10 μM Dantrolene, 1.06±0.12, n=4; $^{\#}p<0.05$, one sample t-test, hypothetical value=1). In the presence of Zn^{2+} (100 μM Zn, *Middle*) and after Zn^{2+} washout (Wash, *Bottom*), effect of blockade for VGCCs and ER-related factors on Zn^{2+}-induced increase in activity (100 μM Zn: Control, 1.81±0.15, n=13; Ni, 1.67±0.33, n=3; Cd, 0.55±0.05, n=3; Nifedipine, 0.98±0.2, n=8; Thapsigargin, 1.46±0.21, n=4; 2-APB, 1.37±0.38, n=6; Caffeine, 1.48±0.35, n=4; Dantrolene, 1.03±0.11, n=4; $p<0.05$, one-way ANOVA; Wash: Control, 1.43±0.2, n=12; Ni, 1.17±0.17, n=3; Cd, 0.53±0.03, n=3; Nifedipine, 1.57±0.07, n=5; Thapsigargin, 1.5±0.0, n=4; 2-APB, 1.52±0.42, n=6; Caffeine, 1.46±0.21, n=4; Dantrolene, 1.28±0.05, n=4; $p>0.05$, one-way ANOVA; $^{*}p<0.05$, $^{**}p<0.01$, Dunnett's post-hoc multiple comparison test for control).

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creases in excitability. These finding suggest that the Zn^{2+}-induced augmentation of firing activities was mediated by high-threshold VGCCs. To determine whether L-type high-threshold VGCCs, which mainly mediate the rhythmic firing of SNc dopaminergic neurons, contribute to the Zn^{2+}-mediated augmentation of firing activities, we examined the Zn^{2+}-mediated changes in firing activity in the presence of the L-type VGCCs antagonist, nifedipine (50 μM) (Fig. 2A, *middle*). In accordance with previous reports [10], nifedipine reduced the spike frequency by 27% (Fig. 2B, *top*), and its presence completely blocked the Zn^{2+}-induced augmentation of firing activities (Fig. 2B, *middle*; 100 μM Zn^{2+}). However, nifedipine had no effect on the Zn^{2+}-mediated increase in firing activities after the Zn^{2+} washout (Fig. 2B, *bottom*, Wash), suggesting that L-type VGCCs are involved in the Zn^{2+}-mediated increase in firing frequency.

We further examined whether the release of Ca^{2+} from intracellular stores is responsible for the Zn^{2+}-induced potentiation of excitability. To deplete intracellular Ca^{2+} stores, the Ca^{2+}-ATPase

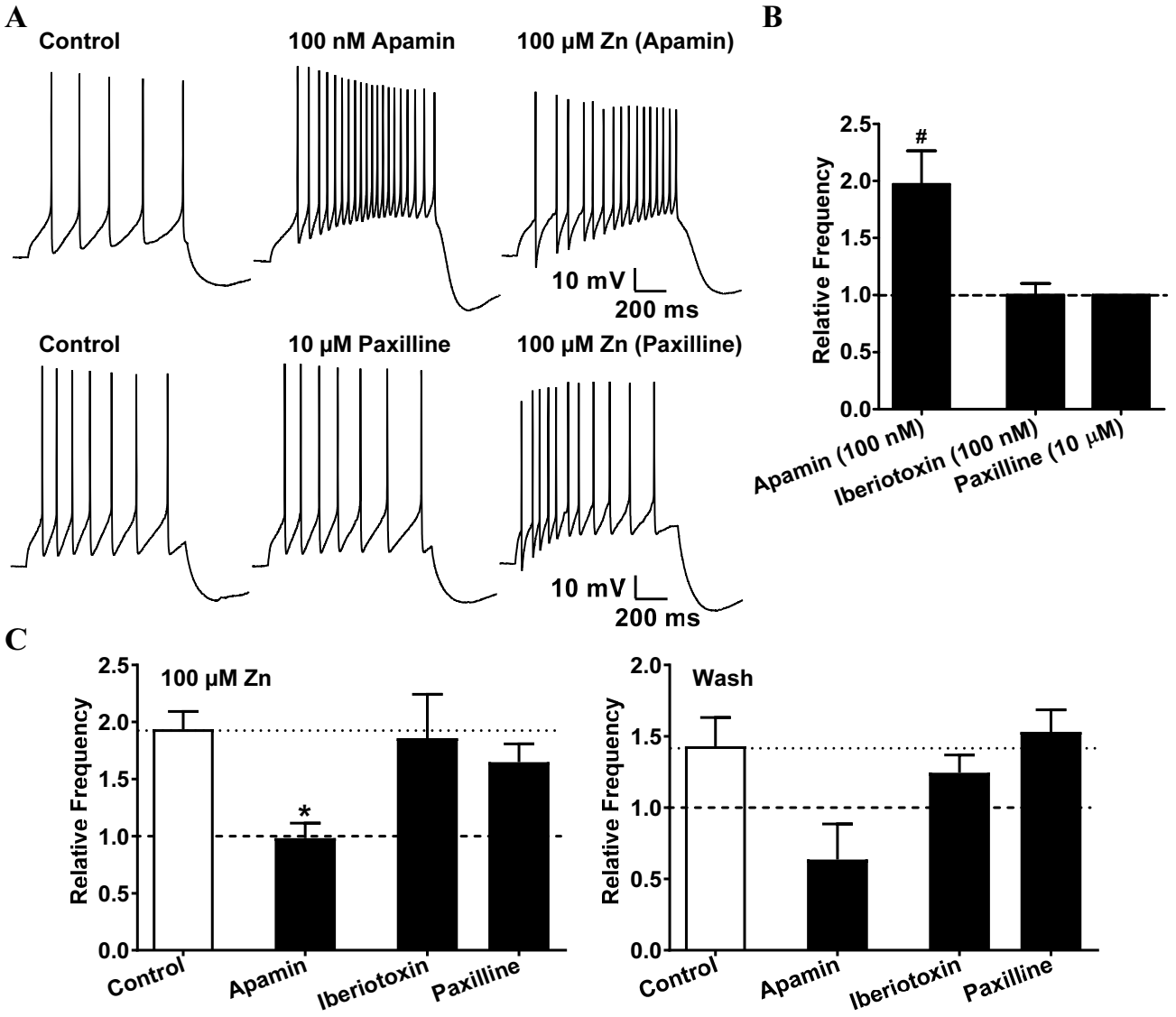

Fig. 3. Involvement of SK channels in the Zn^{2+}-mediated augmentation of excitability in SNc dopaminergic neurons. (A) Current-injected voltage raw traces by a depolarizing pulse (100 pA current injection for 1 s). *Top*, 100 nM Apamin; *Bottom*, 10 μM Paxilline. (B) Spike frequency changes by inhibition of SK channels but not by BK channels (100 nM Apamin, 1.97±0.29, n=4; 100 nM Iberiotoxin, 1.00±0.10, n=4; 10 μM Paxilline, 1.00±0.0, n=4; $^{\#}p<0.05$, one sample t-test, hypothetical value=1). (C) Significant decrease in Zn^{2+}-induced augmentation of activity by apamin (100 μM Zn: Control, 1.81±0.15, n=13; Apamin, 0.98±0.13, n=4; Iberiotoxin, 1.86±0.38, n=4; Paxilline, 1.65±0.16, n=4; $p<0.05$, one-way ANOVA; Wash: Control, 1.43±0.2, n=12; Apamin, 0.64±0.25, n=4; Iberiotoxin, 1.24±0.12, n=4; Paxilline, 1.53±0.15, n=4; $p>0.05$, one-way ANOVA; $^{*}p<0.05$; Dunnett's post-hoc multiple comparison test for control).

inhibitor thapsigargin (1 μM) was added to the internal recording solution. Thapsigargin had no significant effects on firing activities, either in the presence of Zn^{2+}, or after the Zn^{2+} washout (Fig. 2B). This suggested that Ca^{2+} release from the intracellular stores is not responsible for the Zn^{2+}-induced potentiation of excitability. However, because thapsigargin leads to an increase in intracellular free Ca^{2+} by potently inhibiting the ER Ca^{2+}-ATPase, we also tested the channels responsible for this Ca^{2+}-induced Ca^{2+} release from the ER. Two major pathways have been identified for the release of Ca^{2+} from intracellular stores: the IP_3- and ryanodine-sensitive Ca^{2+} stores [26]. To assess the potential role of IP_3Rs in the Zn^{2+}-mediated increase in firing frequency, we used a membrane-permeable IP_3R antagonist, 2-aminoethoxydiphenyl borate (2-APB, 100 μM), which caused a 25% reduction in spike frequency but did not alter the Zn^{2+}-mediated enhancement of firing activities. The release of Ca^{2+} from IP_3Rs or the influx of Ca^{2+} via VGCCs could further trigger Ca^{2+}-induced Ca^{2+} release from the RyRs. To examine the possible role of RyRs in Zn^{2+}-mediated increases in activity, we used caffeine to activate the RyR-sensitive Ca^{2+} stores [27]. For these experiments, we used 5 mM caffeine, which exceeds the K_D of 1.5 mM for caffeine-induced Ca^{2+} release from ER stores [28]. Caffeine had no significant effects on firing activities, either in the presence of Zn^{2+}, or after Zn^{2+} washout (Fig. 2B). Dantrolene, which is a selective membrane-permeable inhibitor of RyRs [29, 30], inhibited the Zn^{2+}-induced increase in spike frequency, but had no effect on firing activity after the Zn^{2+} washout (Fig. 2A, bottom; Fig. 2B). These findings indicated that the release of Ca^{2+} from the RyRs is involved in the augmentation of firing frequency

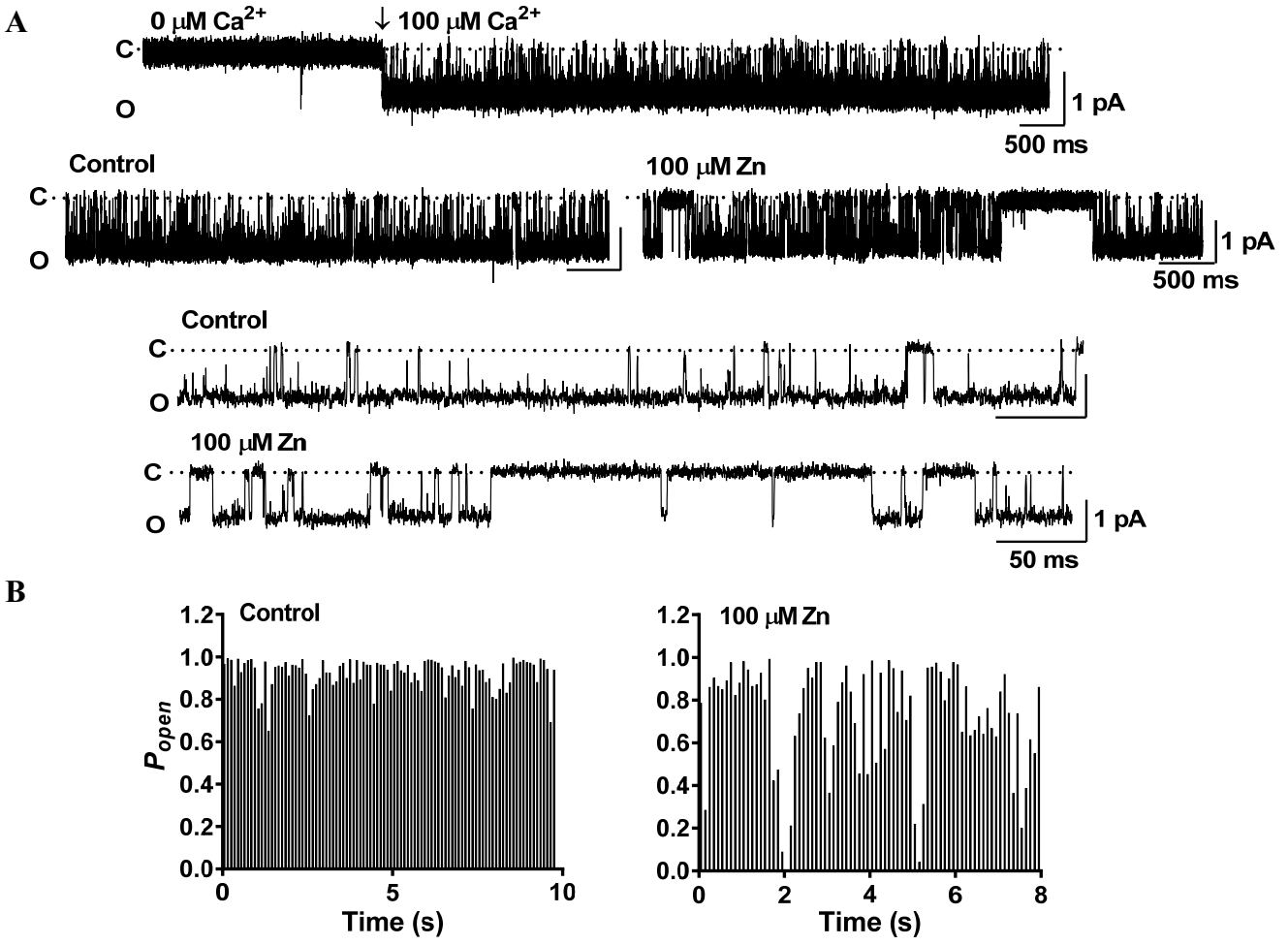

Fig. 4. Inhibition of SK channel by Zn in single channel recording. (A) Inside-out patch single-channel records from a SNc dopaminergic neuron, showing inward SK currents under symmetric potassium conditions. Holding potential was -60 mV, and the calcium concentration at the intracellular patch face was increased to 0.1 mM free calcium as shown by the single channel openings at the top. Middle and bottom panel show SK single-channel current traces in the absence and the presence of 100 μM Zn. The zero current trace is marked by the dashed line (C, closed; O, open). (B) The open probability histogram of an SK channel being open (P_{open}) during 10 s in the absence and the presence of Zn.

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in the presence of Zn^{2+}.

Involvement of SK channels in the zinc-mediated augmentation of excitability

BAPTA and EGTA differ considerably in their Ca^{2+} binding rate constants, with BAPTA being about 150 times faster than EGTA [31]. Therefore, BAPTA is noticeably more effective in preventing the diffusion of free Ca^{2+} away from the entrance site at the plasma membrane. To determine whether the K_{Ca} channels (large-conductance K_{Ca} channels, BK channel; small-conductance K_{Ca} channels, SK channel), which are controlled by local Ca^{2+} signaling domains, are involved in the Zn^{2+}-mediated modulation of excitability in SNc neurons, we examined the effects of BK and SK channels on neuronal excitability in the presence of Zn^{2+}.

The SNc region has low expression of BK channels [32], and shows higher expression levels of the SK3 subunit [33, 34]. In our experiment, apamin (100 nM),—a highly specific blocker of SK channels—increased the excitability in dopaminergic neurons, whereas BK channel blockers like iberiotoxin (100 nM) and paxilline (10 μM) did not induce any changes in excitability (Fig. 3B). These observations supported previous reports [32-34] that apamin-sensitive SK channels are mainly expressed in SNc neu-

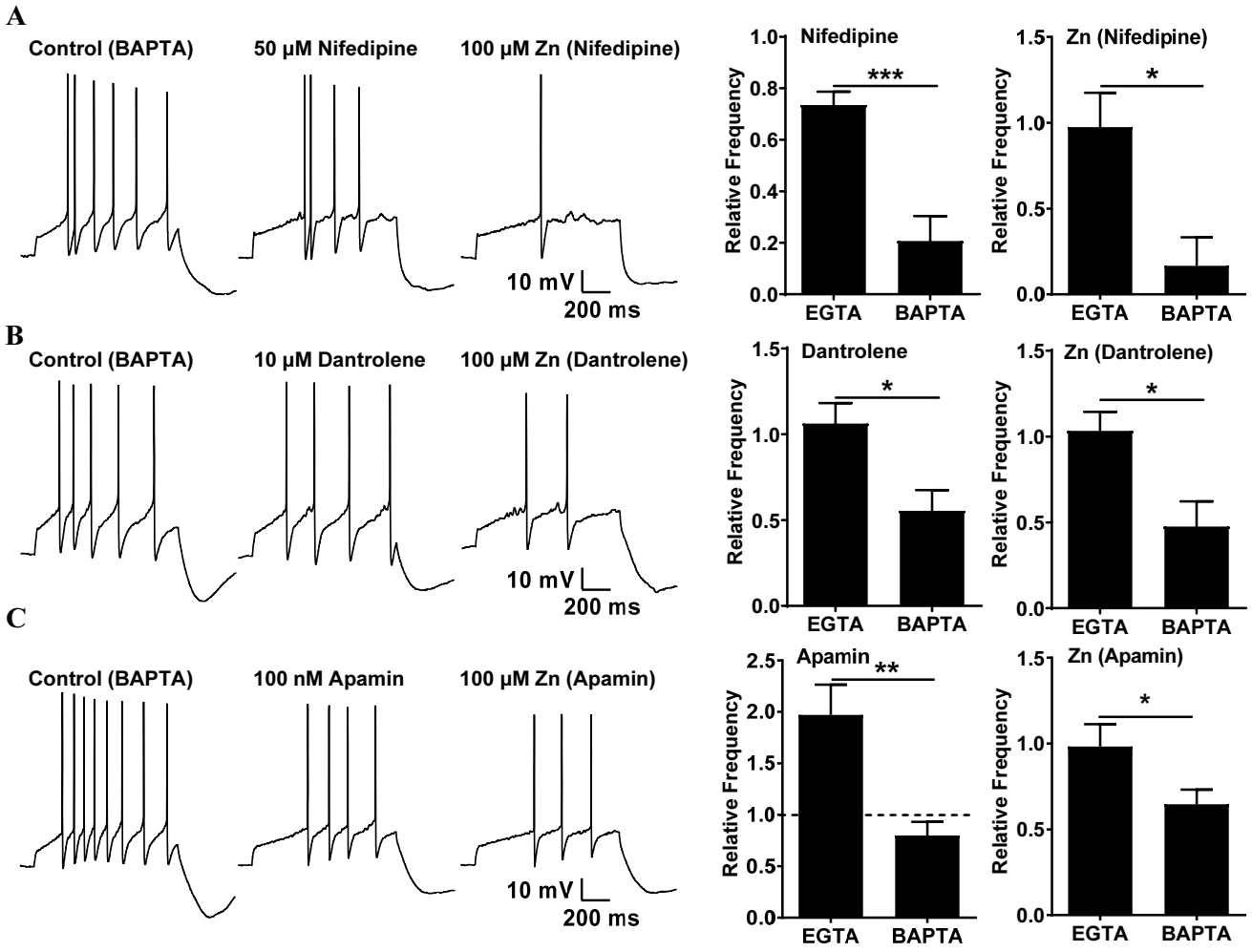

Fig. 5. Differential modulation of the neuronal excitability under EGTA or BAPTA condition by Zn^{2+} in the presence of nifedipine, dantrolene, apamin. *Left*, current-injected voltage raw traces by a depolarizing pulse (100 pA current injection for 1 s) under BAPTA condtion. *Middle*, effect of buffer chelators on the frequency of evoked action potentials. *Right*, effect of buffer chelators on the frequency of evoked action potentials by Zn^{2+}. (A) In the presence of nifedipine. *Middle*, EGTA, 0.73±0.05 Hz, n=8; BAPTA, 0.21±0.09 Hz, n=5; ***p<0.001, unpaired t-test. *Right*, EGTA, 0.98±0.19 Hz, n=8; BAPTA, 0.17±0.17 Hz, n=3; *p<0.05, unpaired *t*-test. (B) In the presence of dantrolene. *Middle*, EGTA, 1.06±0.12 Hz, n=4; BAPTA, 0.55±0.12 Hz, n=8; *p<0.05, unpaired t-test. *Right*, EGTA, 1.03±0.11 Hz, n=4; BAPTA, 0.48±0.14 Hz, n=7; *p<0.05, unpaired t-test. (C) In the presence of apamin. *Middle*, EGTA, 1.97±0.29 Hz, n=4; BAPTA, 0.79±0.13 Hz, n=4; **p<0.01, unpaired t-test. *Right*, EGTA, 0.98±0.13 Hz, n=4; BAPTA, 0.64±0.08 Hz, n=4; *p<0.05, unpaired t-test.

rons, while BK channels are not. To elucidate the effect of Zn^{2+} on SK channels, we investigated whether the Zn^{2+}-mediated increase in firing activity could be blocked by a pretreatment of apamin. Apamin pretreatment significantly attenuated the Zn^{2+}-induced augmentation of excitability, while the BK channel blockers iberiotoxin and paxilline had no effect on it (Fig. 3C). The effects of apamin pretreatment were maintained even after the washout of extracellular Zn^{2+}, suggesting that Zn^{2+} could be acting on an intracellular domain of the SK channels. These results indicated that like apamin, Zn^{2+} was capable of blocking the SK channels, thus inducing an increase in excitability. To confirm the possibility of inhibition of SK channels by Zn^{2+} in SNc neurons, we performed inside-out patch single-channel recording. We found that Zn^{2+} reduced the channel open probability of SK channel, suggesting that Zn^{2+} directly blocks inside SK channels (Fig. 4).

In order to determine how the effect of Zn^{2+} is different in the presence of BAPTA versus EGTA, the aforementioned experiments were conducted with BAPTA as well. Interestingly, in the presence of nifedipine or dantrolene, neuronal activities were lower in the BAPTA condition than in the EGTA condition (Fig. 5A and 5B), and increased neuronal activities were observed only when the SK channels were blocked by apamin in the EGTA condition (Fig. 5C). Moreover, nifedipine, dantrolene, and apamin significantly decreased the neuronal activity of Zn^{2+} under the BAPTA condition. Apamin increased neuronal activity under the EGTA condition and decreased it under the BAPTA condition, in a manner identical to the actions of Zn^{2+} (Fig. 5C).

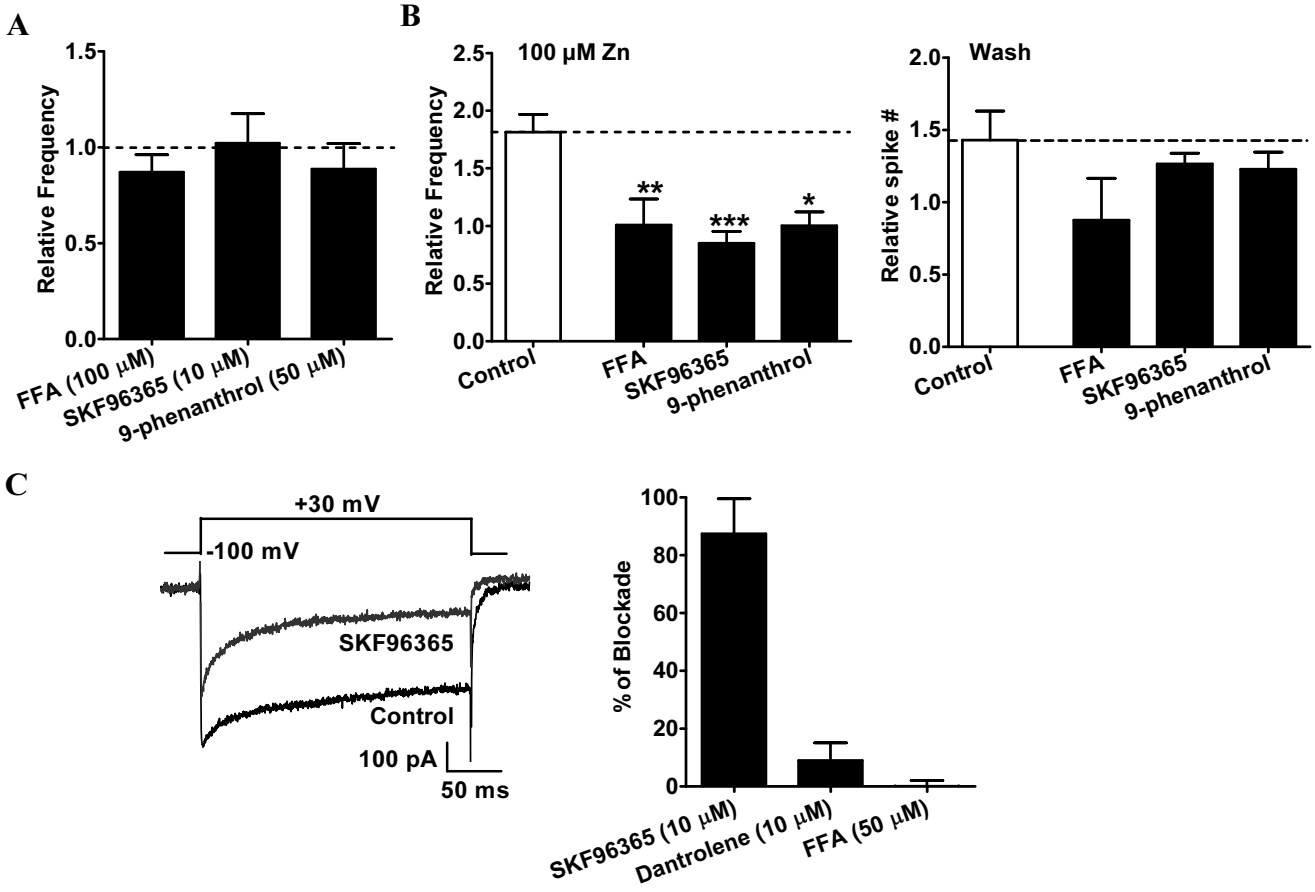

Fig. 6. Involvement of TRPM channels in the Zn^{2+}-mediated augmentation of excitability in SNc dopaminergic neurons. (A) No alteration of spike frequency by the blockade of TRP channels (100 μM FFA, 0.87±0.09, n=6; 10 μM SKF96365, 1.02±0.15, n=5; 50 μM 9-phenathrol, 0.89±0.13, n=4). (B) Significant reduction of Zn^{2+}-induced augmentation of excitability by TRP channel blockers (100 μM Zn: Control, 1.81±0.15, n=13; FFA, 1.01±0.23, n=6; SKF96365, 0.85±0.1, n=7; 9-phenathrol, 1.0±0.12, n=4; $p<0.001$, one-way ANOVA; *$p<0.05$, **$p<0.01$, ***$p<0.001$, Dunnett's post-hoc multiple comparison test for control; Wash: Control, 1.43±0.2, n=12; FFA, 0.88±0.29, n=6; SKF96365, 1.26±0.07, n=4; 9-phenathrol, 1.23±0.12, n=4; $p>0.05$, one-way ANOVA). (C) Significant inhibition of VGCC currents by SKF96365. *Left*, in the voltage-clamp mode, the VGCC current in isolated SNc dopaminergic neurons were elicited by 200-ms step pulses to +30 mV from a holding membrane potential of -100 mV. *Right*, summary of the VGCC current inhibition (10 μM SKF96365, 87.38±12.12 %, n=5; 10 μM Dantrolene, 8.96±6.11 %, n=3; 50 μM FFA, 0.07±2.04 %, n=4).

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Involvement of TRPM channels in the zinc-mediated augmentation of excitability

The tonic Ca^{2+} entry pathways that maintain basal cytosolic Ca^{2+} levels consist mainly of TRP channels, and contribute to the generation of spontaneous firings [10]. Therefore, the dopaminergic neurons in which Zn^{2+} enhanced firing activities were first tested for their response to flufenamic acid (FFA), a broad-spectrum TRP channel blocker. The application of FFA (100 μM) had no effect on firing activity, but it completely prevented the Zn^{2+}-induced increase in firing activity (Fig. 6), suggesting that the potentiation of firing activity by Zn^{2+} occurred via TRP channels. On application of 10 μM of SKF96365—a widely-used blocker of canonical TRP (TRPC) channels—the potentiation of firing activity by Zn^{2+} was strongly inhibited (Fig. 6), but these effects were reversed upon the washout of SKF96365. In some preparations, SKF96365 also blocks VGCCs [35]. To determine whether the antagonists for Ca^{2+}-related channels directly affect VGCCs, we tested SKF96365, dantrolene, and FFA on the VGCCs of acutely isolated SNc neurons using voltage-clamp recording (Fig. 6C). VGCC currents were elicited from isolated SNc neurons held at -100 mV by applying +30 mV, which activated all the VGCCs as expected. SKF96365 (10 μM) substantially reduced the steady-state VGCC currents by 87%, and dantrolene (10 μM) reduced the steady-state VGCC currents by 9%, but FFA (50 μM) did not significantly inhibit whole-cell steady-state VGCC currents (Fig. 6C). Therefore, we concluded that the inhibition of the Zn^{2+}-induced firing potentiation by SKF96365 is mainly mediated via a VGCC inhibition, not a TRPC inhibition.

Melastatin-like TRP (TRPM) channels, which are directly activated by intracellular Ca^{2+}, and conduct Na^{+} ions to further depolarize cells [36], are expressed in the neurons of the SNc [37]. To examine the possibility that TRPM channels are involved in the Zn^{2+}-induced potentiation of excitability in SNc neurons, we applied 9-phenanthrol, a drug that selectively blocks TRPM channels [38]. 9-phenanthrol (50 μM) completely eliminated the Zn^{2+}-induced amplification of firing activity in the tested neurons. These effects were reversed upon the washout of 9-phenanthrol, suggesting that TRPM channels could play an important role in the enhancement of firing activity by Zn^{2+}.

DISCUSSION

Zn^{2+} is present in high concentration in the SN region of the parkinsonian brain [39-41], and can increase or inhibit the intrinsic firing activity of rat SNc dopaminergic neurons depending on the intracellular Ca^{2+} buffering capacity, suggesting the involvement of cytosolic Ca^{2+}-dependent signaling processes. To elucidate the effect of Zn^{2+} on the excitability of dopaminergic SNc neurons, this study investigated its action on cytosolic Ca^{2+}-dependent processes in relation to the intracellular Ca^{2+} buffering capacity.

The prominent results of this study can be summarized as follows: (1) In the depolarized state, Zn^{2+} increases the tonic activity during intracellular dialysis with EGTA, but decreases firing activity in the presence of BAPTA. (2) The augmentation of firing activity in the presence of Zn^{2+} is blocked by the RyR blocker dantrolene, and by the VGCC antagonists nifedipine and Cd^{2+}, but not by the IP_3R antagonist 2-APB. (3) The Zn^{2+}-mediated potentiation of firing activity is eliminated by the blockade of TRPM channels with FFA and 9-phenanthrol. (4) Zn^{2+} directly reduced the channel open probability of SK channels. Apamin—an SK channel inhibitor—enhances the firing activity, and its effect is attenuated by intracellular dialysis with BAPTA. Apamin blocks the augmentation of firing activity not only in the presence of Zn^{2+}, but also after the washout of Zn^{2+}.

We propose some hypothetical mechanisms for Zn^{2+}-mediated augmentation of excitability leading to Ca^{2+} signaling processes (Fig. 7). When SK channels are blocked, the firing activity of neurons can increase. Apamin is very similar in its actions compared to Zn^{2+}. Apamin enhances tonic activity in the depolarized state when the cell is intracellularly dialyzed with EGTA, whereas apamin attenuates the firing activity in the presence of intracellular BAPTA (Fig. 5C). This is in agreement with a high expression of SK3 channels, but not BK channels in SNc neurons [32, 34]. Iberiotoxin and paxilline, which are BK channel antagonists, have no effect on the firing activity in SNc neurons (Fig. 3C). In the presence of apamin, the Zn^{2+}-induced enhancement of firing activity is completely blocked and this blockage is sustained even after Zn^{2+} removal from the bath solution, suggesting that Zn^{2+} possibly acts on SK channels in both the extracellular and intracellular domains. In inside-out patch single-channel recording, Zn^{2+} directly reduced the SK channel open probability (Fig. 4). These results are consistent with the previous studies that Zn^{2+} has been reported to directly inhibit the activation of K_{Ca} channels in other preparations such as hippocampal neurons and motor neurons [42, 43]. Therefore, we suggest that Zn^{2+} enhances the firing activity of SNc neurons via the blockade of SK channels by Zn^{2+}.

Cytosolic Ca^{2+} triggers a wide variety of Ca^{2+}-dependent signaling events and reaction cascades, and its levels rise only for short periods of time and at spatially restricted domains. Such local Ca^{2+} signaling domains are generated by a variety of Ca^{2+} buffer systems, such as BAPTA and EGTA, which limit the diffusion of Ca^{2+} ions that have entered the cells through VGCCs or Ca^{2+}-permeable channels [44, 45]. BAPTA, but not EGTA, interferes effectively with Ca^{2+} signaling processes in Ca^{2+} nanodomains, i.e.,

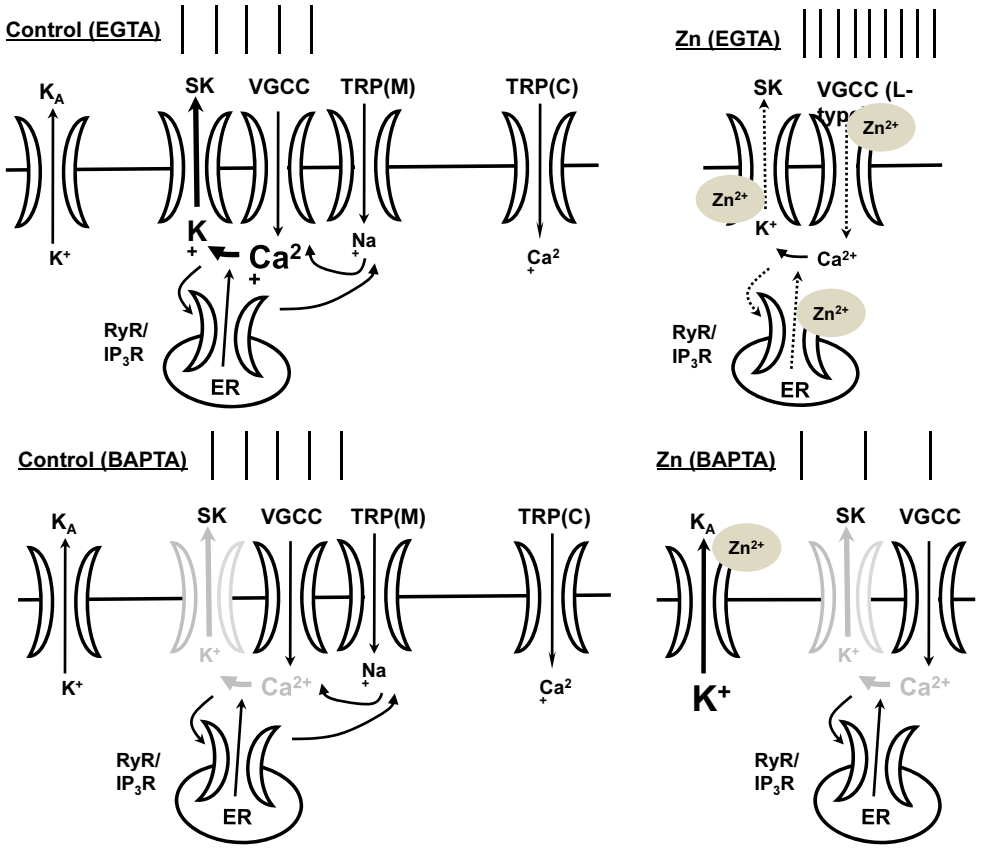

Fig. 7. A scheme to illustrate spatial relationships among VGCC, TRP channel, RyR and SK channel and Zn^{2+} action in different Ca^{2+} chelator condion. Intracellular Ca^{2+} sources are extracellular Ca^{2+} influx via VGCC, TRP channel and Ca^{2+}-induced Ca^{2+} release from RyR/IP3R in ER. Ca^{2+} concentration increases also induce the activation of TPRM channel amplifying cytosolic Ca^{2+} increases. EGTA condition, internal Ca^{2+} plays a key role in neuronal activity, and their blockades of Ca^{2+}-related channels as a Ca^{2+} source and SK channel as a Ca^{2+} sensor act as an major factor in Zn^{2+}-induced augmentation of neuronal activity. BAPTA condition, internal Ca^{2+} action is excluded, and thus KA channel acts as key factor in Zn^{2+}-induced alteration of neuronal activity (thick arrow, increase; thin arrow, normal function; broken arrow, inhibition; gray channel & arrow, nonfunctional).

within 20~50 nm of the Ca^{2+} source, while both modulate Ca^{2+} signaling processes equally in Ca^{2+} microdomains, i.e. between 50 nm to a few hundred nm from the Ca^{2+} source [5]. The ability of Zn^{2+} to enhance the excitability in the presence of EGTA, but not of BAPTA, suggests that rapid local changes in Ca^{2+} are necessary for the augmentation of firing activity. In SNc neurons, one potential mechanism for rapid Ca^{2+} dynamics causing Zn^{2+}-mediated increases in firing activity is the co-localization of VGCCs or RyRs as Ca^{2+} sources, and SK channels as Ca^{2+} sensors. If those VGCCs or RyRs and SK channels were located in Ca^{2+} nanodomains, Zn^{2+} would have no effect on neuronal excitability when using a BAPTA-loaded pipette, because BAPTA would rapidly decrease the free Ca^{2+} concentration, thus inactivating the SK channel. However, in the presence of EGTA, the reduction of SK channel activation by Zn^{2+} results in an augmentation of excitability. Because Zn^{2+} increases the excitability in experiments with an EGTA-containing, but not a BAPTA-containing, internal solution, we suggest that VGCCs or RyRs and SK channels are located in Ca^{2+} nanodomains, and Zn^{2+} possibly attenuates SK channel activity. In the BAPTA condition, due to a decrease in the levels of free Ca^{2+}, Zn^{2+}-induced neuronal activity would be determined by the action of KA channels (Fig. 7).

Because the Zn^{2+}-mediated potentiation of firing activity in SNc neurons was completely blocked by pretreatment with nifedipine, Cd^{2+}, or dantrolene (Fig. 2), we conclude that the Zn^{2+}-mediated augmentation of activity requires an increase in cytosolic Ca^{2+} concentration via the activation of L-type VGCCs and RyRs. Zn^{2+} may directly block the SK channels, and/or indirectly reduce SK channel activity via a blockade of Ca^{2+} channels such as the VGCC or RyR/IP_3R, thus attenuating the cytosolic Ca^{2+} concentration. Zn^{2+} significantly reduces the VGCC currents of SN neurons [46]. A plausible explanation for the observed Zn^{2+}-induced potentiation of neuronal excitability is the indirect inhibition of SK channels caused by a decrease in Ca^{2+} influx due to the inhibition of

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VGCCs or RyRs by Zn^{2+}.

TRPM channels, which are activated by cytosolic Ca^{2+} released from IP_3Rs on the ER membrane, further depolarize the neurons by conducting Na^+ ions, and induce a Ca^{2+} influx by activating the VGCCs. When applied extracellularly, 2-APB—a membrane-permeable blocker of intracellular IP_3Rs—augments Ca^{2+} entry through store-operated channels (SOCs) at lower concentrations (<10 μM), and blocks TRPC channels at higher concentrations (>30 μM) [47-51]. The presence of 100 μM 2-APB failed to inhibit the Zn^{2+}-mediated augmentation of firing activity, suggesting that the potentiation of neuronal activity by Zn^{2+} is not mediated by IP_3Rs and TRPC channels. SKF96365, another well-known antagonist of TRPC channels, blocks SOCs and TRPC channels at concentrations of 25~100 μM [52-54], and in some preparations also blocks VGCCs [35]. SKF96365 was a potent blocker of VGCCs in this study as well (Fig. 4C). Because VGCC antagonists, such as nifedipine or Cd^{2+} (Fig. 2B), completely blocked the Zn^{2+}-mediated increase in firing activity, we suggest that the prevention of Zn^{2+} action by SKF96365 is exclusively mediated by VGCC inhibition, and not by the blockade of TRPC channels. 9-phenanthrol blocks TRPM4 channels in a human embryonic kidney cell expression system with a half-maximal inhibitory concentration (IC_{50}) of 16.7 μM under conventional whole-cell patch-clamp conditions, and does not alter the activity of TRPC channels, BK channels, inward-rectifying K^+ channels, or VGCCs [55]. Therefore, FFA and 9-phenanthrol halt the Zn^{2+}-mediated potentiation of firing activity by inhibiting the TRPM—most likely, TRPM4—channels. We suggest that the Zn^{2+}-mediated inhibition of TRPM channels causes a decrease in SK activity via the reduction of cytosolic Ca^{2+}, which contributes to the observed augmentation of firing activity in the SNc neurons.

In summary, we propose that (1) extracellular Zn^{2+} reduces the cytosolic Ca^{2+} concentration not only via the blockade of VGCCs, but also by reducing the Ca^{2+}-mediated inactivation of RyRs/TRPM channels, resulting in a decrease in the activation of SK channels, and (2) Zn^{2+} directly blocks SK channels via interactions with an intracellular domain. This suggests that in the SNc, the blockade of SK channels by Zn^{2+} directly and/or indirectly induces the Zn^{2+}-mediated augmentation of excitability.

Several Ca^{2+}-binding proteins—or Ca^{2+} buffering proteins, including calretinin, calbindin and parvalbumin—are found throughout the central nervous system, including in the midbrain dopaminergic neurons, and help maintain a tight regulation of Ca^{2+} signals [56-58]. These high-affinity Ca^{2+}-binding proteins act as buffers to prevent abrupt changes in the intracellular Ca^{2+} concentration [59, 60], and this is essential in preventing cell dysfunction and excitotoxic damage caused by high intracytoplasmic concentrations of Ca^{2+} [49, 61]. Some reports emphasize the role of Ca^{2+}-sensing receptor or Ca^{2+}-binding proteins/ Ca^{2+}-buffering protein in pathophysiology of glaucoma and vulnerability to traumatic noise [62, 63]. The pathology of Parkinson's disease involves a significant loss of parvalbumin-immunoreactivity in SN neurons [64], suggesting that this Ca^{2+}-buffering protein plays a crucial role in maintaining normal Ca^{2+} signaling processes and Ca^{2+} homeostasis. Based on the results our experiments using EGTA and BAPTA, we propose that Zn^{2+} could increase the excitability of SNc neurons in pathological conditions involving a decrease in the activity of Ca^{2+}-binding proteins, similar to the EGTA condition, whereas Zn^{2+} could decrease the firing activity in physiological conditions involving an increased activity of Ca^{2+}-binding proteins, similar to BAPTA condition. This suggests that exogenous or endogenous factors such as Zn^{2+} could differentially modulate the excitability of neurons, depending on the physiological or pathological state of synaptic as determined by the Ca^{2+}-buffering capacity. By attenuating Ca^{2+} influx into the cytosol, Zn^{2+} may also compensate for the loss of SNc dopaminergic neurons during the development of Parkinson's disease [65], thus maintaining normal information processes in the basal ganglia until a critical mass of dopaminergic neurons are generated.

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