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(RESEARCH ARTICLE)

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Extracellular calcium induces neurite outgrowth through calcium-sensing receptors in PC12 HS cells

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Abstract

PC12 cells have been used extensively to study neuronal differentiation and neurite outgrowth. However, the effects of extracellular Ca2+ on PC12 cells have not yet been sufficiently evaluated. In this study, we investigated the effect of extracellular calcium using PC12 HS cells, which have high sensitivity to nerve growth factor (NGF). The addition of Ca^{2+} (1 mM) to the medium induced neurite outgrowth and increased the intracellular calcium concentration. In contrast, the calcium-sensing receptor antagonist NPS2143 suppressed neurite outgrowth and increased the intracellular calcium concentration, suggesting that extracellular calcium promotes neurite outgrowth through calcium-sensing receptors. An increase in extracellular calcium concentration increased phosphorylation of Akt and cyclic AMP response element-binding protein (CREB). Semi-quantitative RT-PCR showed that elevated extracellular calcium increased the mRNA expression of brain-derived neurotrophic factor (BDNF) and platelet-derived growth factor (PDGF), which are regulated by CREB activation. Our results suggest that elevated extracellular calcium can promote the phosphorylation of Akt and CREB through calcium-sensing receptors, resulting in the promotion of neurites. PC12 HS cells are a useful model to investigate the regulation of extracellular calcium via calcium-sensing receptors in neuronal cells.

Keywords: Calcium-sensing receptor; Extracellular calcium; Neurite extension; PC12HS

1. Introduction

Ca^{2+} serves as an important intracellular signal for cellular processes such as differentiation and apoptosis. The Ca^{2+} concentration in the cytosol is maintained at about 10^{-6}–10^{-7} M, which is 10^4-fold lower than the extracellular Ca^{2+} concentration (approximately 1–2.5 mM). An increase in intracellular Ca^{2+} levels leads to various types of cellular responses. Cells also respond to subtle changes in extracellular Ca^{2+} concentration, which can modulate the balance between the proliferation and differentiation of cells, including chondrocytes and fibroblasts [1-3]. This response is mainly mediated by calcium-sensing receptors (CaSRs) that respond to changes in extracellular Ca^{2+} concentration. CaSRs are widely expressed in every tissue, including the brain, but their function in neurons is unclear. Several studies have shown that CaSRs play a significant role during the differentiation of neuronal progenitor cells and regulate axonal and dendritic growth in both central and peripheral nervous systems [4, 5]. Vizart et al. reported that activating the CaSRs in cultured superior cervical ganglion (SCG) neurons with elevated levels of extracellular calcium enhances axon growth in the presence of NGF but not in the absence of NGF [4]. The requirement for NGF in CaSR-promoted axonal growth complicates research on the roles of CaSRs and extracellular calcium. In a study using PC12 cell lines, extracellular calcium was found to regulate neurite outgrowth in the presence of NGF, but not in the absence of NGF, although it is unclear whether the regulation occurs via CaSRs [6]. To observe the effects of extracellular calcium and neurite outgrowth in neuronal cells, we used PC12 HS cells, which are sensitive to NGF. In this study, we examined the effects of extracellular calcium on neurite extension and signal transduction in PC12 HS cells.

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2. Material and methods

2.1. Materials

Antibodies against β-actin, phosphorylated cAMP response element binding protein (p-CREB), phosphorylated protein kinase B (p-Akt), and phosphorylated extracellular receptor kinase (p-ERK) were purchased from Biorbyt (San Francisco, CA, USA).

2.2. Cell culture

The PC12 HS cells (JCRB0733) were purchased from the Japanese Collection of Research Bioresource Cell Bank (Osaka, Japan). PC12 HS cells differentiate into neurons. The cells were maintained in Roswell Park Memorial Institute medium (RPMI) 1640 supplemented with 10% fetal bovine serum (FBS) and 5% horse serum. The calcium concentration in RPMI1640 medium was 0.43 mM. For differentiation, nerve growth factor (NGF) was added (2 ng/ml). To investigate the effect of extracellular calcium, PC12 HS cells were seeded at a density of 1×10^4 cells per 24-well plate, coated with a cell matrix (Nitta gelatin, Japan). After 24 h, $CaCl_2$ was added at the indicated concentrations in the presence or absence of the CaSR antagonist, NPS-2143. After 48 or 72 h, neurite length was measured in at least four randomly selected areas and expressed as the mean neurite length. Cell viability was estimated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and lactate dehydrogenase (LDH) assay, as described previously [7].

2.3. Measurement of intracellular calcium

Intracellular calcium levels were measured using Fluo3-AM. PC12 HS cells were seeded at a density of 1.0×10^4 cells and incubated for 24 h. The medium was replaced with loading medium consisting of 20 mM Hepes-NaOH (pH 7.0), 115 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, and 13.8 mM glucose and was loaded with fluo3-AM (4 μM) for 60 min at 37 ℃. The loading medium was removed, and the cells were washed with PBS three times. Loading medium (200 μl) was added and incubated for 60 min at 37 ℃, calcium was added at 1 mM in the presence or absence of CaSR antagonist, the fluorescence change was photographed every 10 s using confocal microscopy (488 nm excitation; 525 nm emission), and the fluorescent intensity was quantitated.

2.4. Electrophoresis

An SDS sample solution containing 2% SDS, 20 mM Tris-HCl (pH 7.5), 1 mM 2-mercaptoethanol, 10% glycerol, and bromophenol blue was added to the samples. SDS-PAGE was performed according to the method described by Leammli [8].

2.5. Western blotting

After PC12HS cells were incubated in the absence or presence of calcium for 60 min, they were homogenized in 2% SDS and centrifuged at 14,000 × *g* for 5 min. The cell extracts were then subjected to SDS-PAGE on a 12.5% gel. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. The membrane was then blocked with 5% skim milk (w/v) in Tris-buffered saline containing 0.5 M NaCl, 20 mM Tris HCl (pH 7.5), and 0.05% Tween 20 (solution A) for 2 h at room temperature. The membrane was incubated with β-actin, p-CREB, p-Akt, or p-ERK primary antibodies overnight, followed by incubation with an alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibody for 2 h. The signals were developed using nitroblue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate. Protein band intensities were measured using ImageJ software.

2.6. Semi-quantitative RT-PCR analysis

After PC12HS cells were incubated in the absence or presence of calcium, the total RNA was purified using RNAiso Plus (Takara, Shiga, Japan) according to the manufacturer's protocol. After first-strand cDNA synthesis from 20 μg of total RNA using oligo (dT) primers, PCR was carried out using 0.05 μg cDNA template and specific sense and antisense primers of actin, brain-derived neurotrophic factor (BDNF), platelet-derived growth factor (PDGF), and tau in a final volume of 25 μl. The PCR primer sequences are listed in Table 1. The expression of the target genes was normalized to the mean expression level of β-actin. The intensities of the amplified bands were estimated using ImageJ software. The mRNA expression levels were normalized to actin mRNA levels. The amplification cycles were determined based on the relationship between the amount of PCR product detected and the cycle number.

Table 1 Primer sequences

Gene	sequence (5`to3`)
SOD	F-GGCCAAGGGAGATGTTACAA
	R-GCTTGATAGCCTCCAGCAAC
catalase	F-GCGAATGGAGAGGCAGTGTAC
	R-GAGTGACGTTGTCTTCATTAGCACTG
HO-1	F-ACTTTCAGAAGGGTCAGGTGTCC
	R-ACTTTCAGAAGGGTCAGGTGTCC
TrkA	F-GCATGTCAACAACGGGAACT
	R-TGGGTCTCTTGATGTGCTGT
PDGF	F-CATCGGCCAACTTCTTGACC
	R-CTCACATCCGTCTCCTCCTC
BDNF	F-AGCCTCCTCTGCTGTTTCTGCTGGA
	R-CTTTTGTCTATGCCCCTGCAGCCTT
β-actin	F-CCTCTATGCCAACACAGTGC
	R-CCTGCTTGCTGATCCACATC

2.7. Statistical analysis

Each experiment was performed two or three times. Experiments were performed in duplicate or in triplicate. Data from at least four data points were combined and expressed as the mean and standard deviation (SD). Each experiment was performed at least thrice. Data were analyzed using one-way analysis of variance (one-way ANOVA) followed by Tukey's multiple-comparison test.

3. Results

3.1. Extracellular calcium induces neurite outgrowth.

Koike et al. reported that extracellular calcium alone did not stimulate neurite outgrowth in PC12 cells [6]. Therefore, we used PC12 HS cells that were sensitive to NGF. First, we examined toxicity against extracellular calcium in PC12 HS cells using the MTT assay and by determining LDH activity (Figure 1). Although the addition of 5 mM calcium to the medium decreased the number of viable cells, the addition of 1 mM calcium did not decrease the number of viable cells or increase LDH activity in the medium. Therefore, calcium concentrations below 1 mM were used in the present study. To determine the effect of extracellular calcium on neurite outgrowth of PC12 HS cells, PC12 HS cells were cultured in RPMI 1640 medium supplemented with various concentrations of Ca^{2+} (0, 0.2, and 1 mM) from calcium chloride for 72 h. Cultures were photographed using phase contrast microscopy, and neurite length was measured as described in the Materials and Methods. Elevated concentrations of extracellular calcium-induced neurite outgrowth compared to that of the control (Figure 2). Neurite length was increased by about 1.4-fold after the addition of 0.2 mM calcium and about 1.6-fold after the addition of 1 mM calcium compared to that of the control. In contrast, NGF promoted neurite outgrowth by more than 2-fold compared to that of the control. This result was also supported by the mRNA expression levels of tau protein, which is expressed in the neurites. We used the addition of 1 mM calcium in the following experiments because it induced distinct neurite elongation.

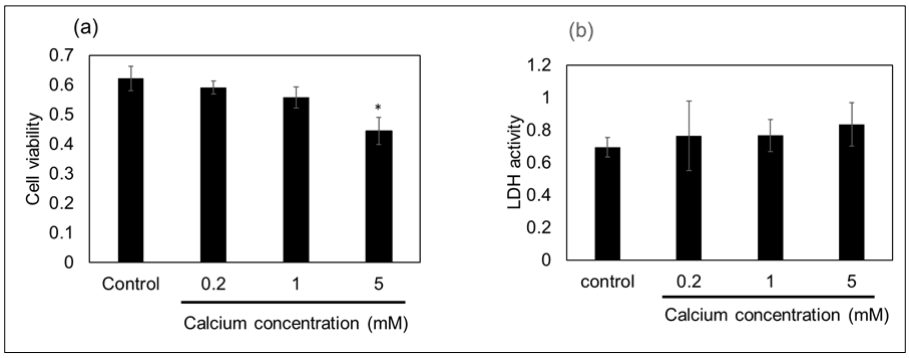
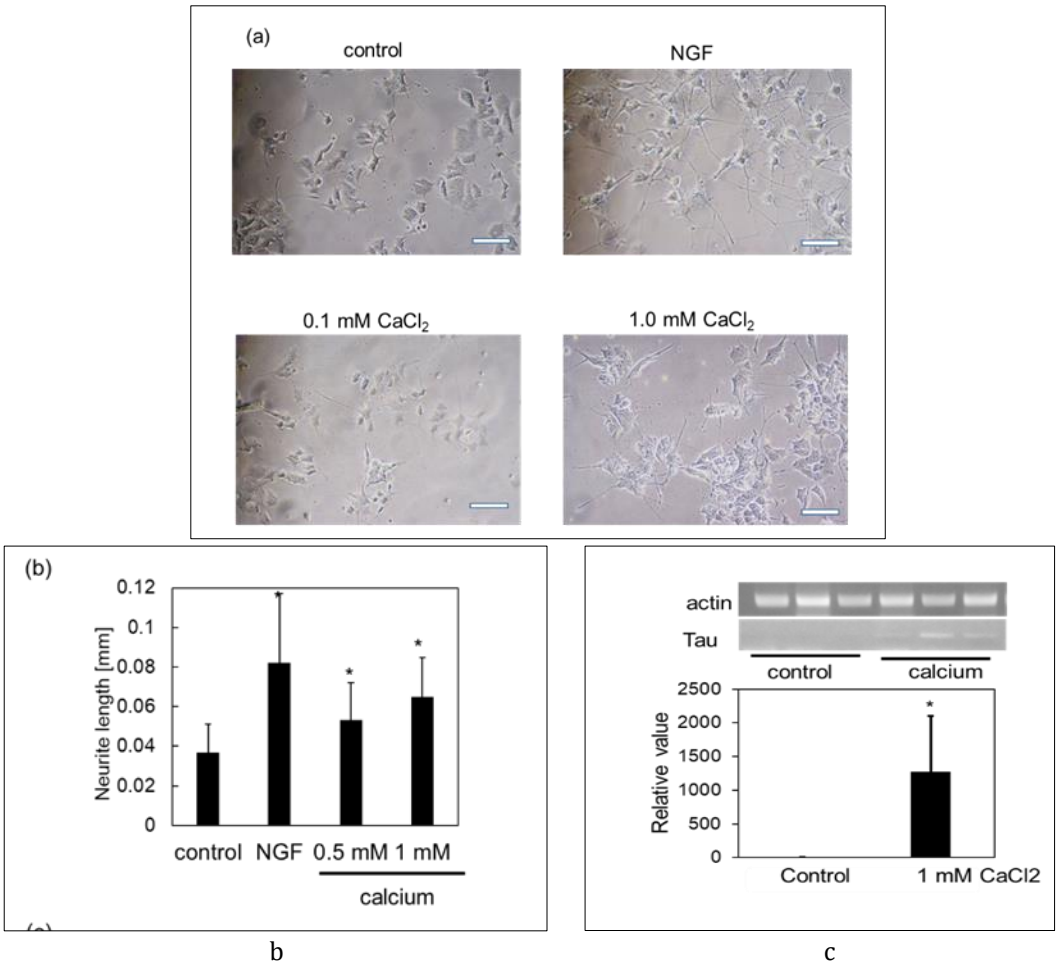


Figure 1 Effect of extracellular calcium on neuronal injury. PC12 HS cells were treated with vehicle (control) or 0.2, 1, or 5 mM $CaCl_2$. After 48 h, the MTT assay was performed, and LDH activity was measured. The difference is considered statistically significant at *p≤ 0.05 versus the control.

3.2. Elevated concentration of extracellular calcium increases intracellular calcium concentration



b c

Figure 2 Effect of elevated extracellular calcium on neurite outgrowth. PC12 HS cells were treated with vehicle (control), 2 mM NGF, 1 Mm $CaCl_2$, or 5 mM $CaCl_2$. (a) After 72 h, cells were examined under phase contrast microscopy and photographed. (b) Neurite length was measured in at least 3 randomly selected areas (n=45), and data are expressed as the mean ± standard deviation (SD). Scale bars show 500 μm. Statistical significance was determined by ANOVA. The difference is considered statistically significant at *p≤0.05 versus the control.

To investigate the mechanism of action of neurite outgrowth, we estimated intracellular calcium concentration using Fluo3-AM because an increase in intracellular calcium concentration has been reported to induce neurite outgrowth [9, 10]. After the addition of 1 mM calcium to the medium, changes in intracellular calcium concentrations were

investigated for 5 min. Intracellular calcium increased by approximately 20-fold compared with the basal level after 5 min (Figure 3).

To investigate whether the activation of CaSRs by extracellular calcium led to an increase in intracellular calcium, we estimated the effect of the CaSR antagonist NPS2143 on the increase in intracellular calcium (Figure 3). The CaSR antagonist, NPS2143, significantly suppressed the increase in intracellular calcium, suggesting that the CaSR contributes to the increase in intracellular calcium in PC12 HS cells due to elevated extracellular calcium. Next, to investigate whether CaSR is involved in neurite outgrowth induced by elevated extracellular calcium, the effect of the CaSR antagonist NPS2143 on neurite outgrowth was estimated (Figure 3). Co-treatment with 1 mM calcium and the CaSR antagonist NPS2143 inhibited neurite outgrowth compared with treatment with calcium alone (Figure 3). To further confirm that CaSR was involved in neurite outgrowth, the effect of the CaSR agonist spermidine on neurite outgrowth was investigated (Figure 4). Spermidine also induced neurite outgrowth compared to the control, and NPS2143 inhibited neurite outgrowth. These results support the hypothesis that elevated extracellular calcium increases the intracellular calcium concentration via CaSRs in PC12 HS cells, leading to neurite extensions.

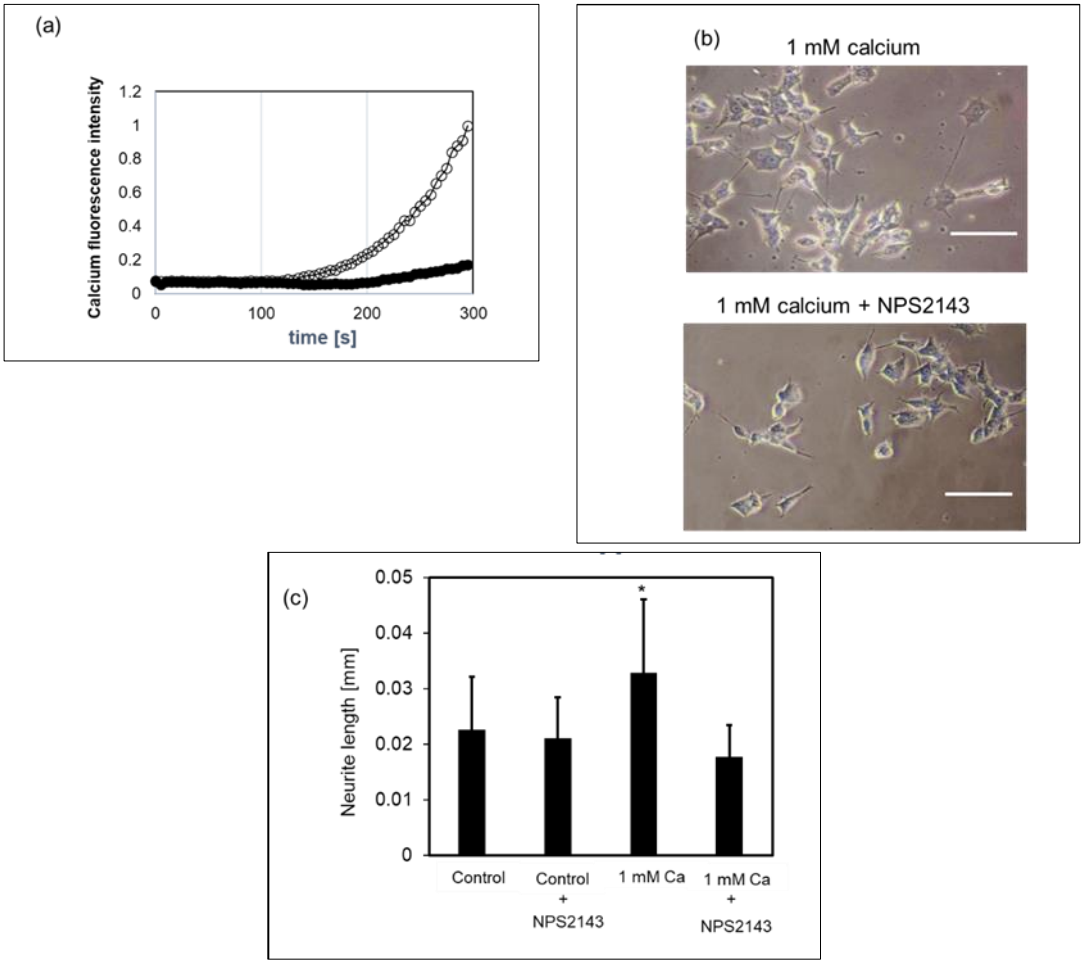


Figure 3 Effect of elevated extracellular calcium on intracellular calcium. (a) PC12 HS cells were treated with 1 mM $CaCl_2$, in the presence or absence of 4.5 μM CaSR antagonist NPS2143. (a) The change in fluorescent intensity was measured every 10 s under confocal microscopy, and the relative fluorescent intensity in the presence of (closed circles) or absence (open circles) of NPS2143 was plotted. (b) After 72 h, photographs were taken under phase contrast microscopy. (c) Neurite length was measured in at least 3 randomly selected areas (n= 45), and data are expressed as the mean ± standard deviation (SD), Scale bars represent 500 μm. The difference is considered statistically significant at *p≤0.05 versus the control.

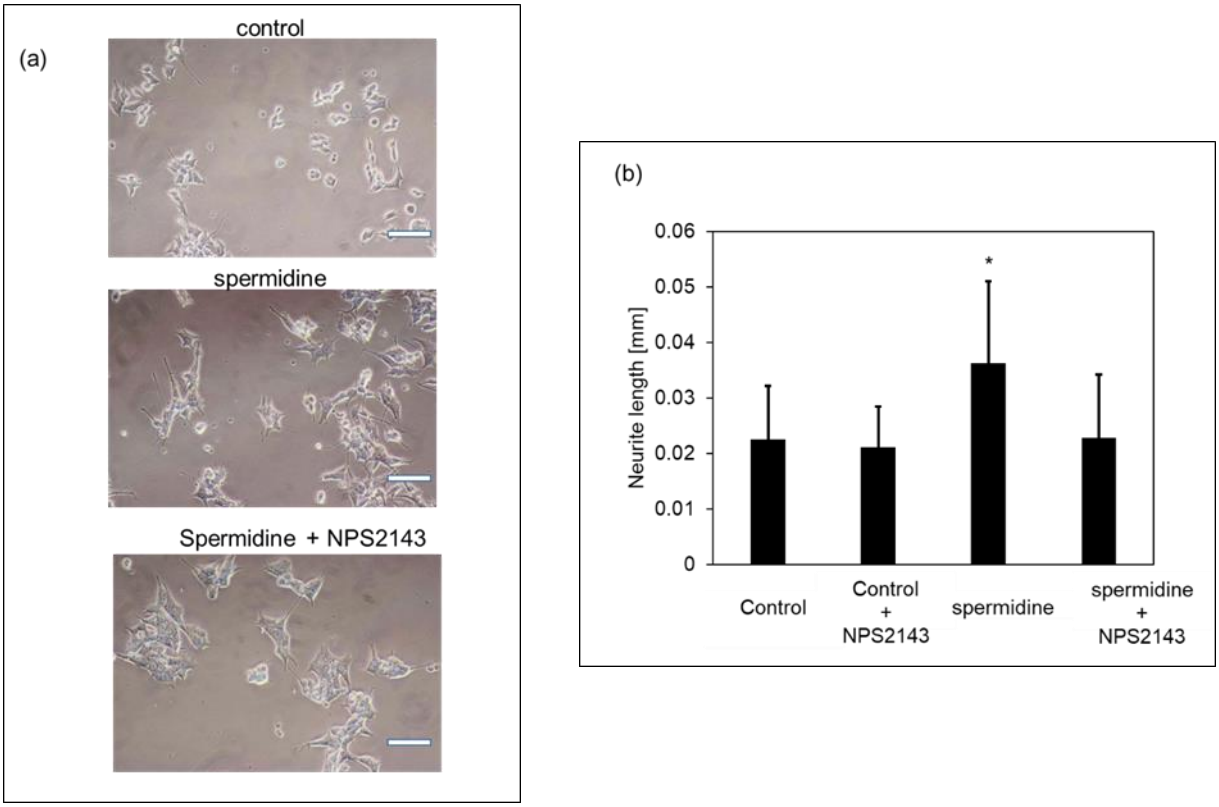


Figure 4 Effect of CaSR agonist spermidine on neurite outgrowth and the expression of CaSR. (a) PC12 HS cells were treated with 0.25 mM spermidine in the presence or absence of 4.5 μM CaSR antagonist NPS2143. After 72 h, photographs were taken and (b) Neurite length was measured. Scale bars represent 500 μm. Data are expressed as the mean ± SD. Statistical significance was determined by ANOVA. The difference is considered statistically significant at *p≤0.05 versus the control.

3.3. Effect of flunarizine on neurite outgrowth and intracellular calcium in the presence of elevated extracellular calcium

Activation of the CaSR has been proposed to activate neuronal potassium channels (11) and an unusually non-selective channel in neuronal somata (11). To investigate whether calcium entry via calcium channels contributes to neurite outgrowth by extracellular calcium, we examined the effect of the calcium channel blocker flunarizine, a nonselective calcium channel blocker (12). The addition of flunarizine inhibited extracellular calcium-induced neurite outgrowth and increased intracellular calcium levels (Figure 5), suggesting that the activation of the CaSR leads to the entry of extracellular calcium via calcium channels, resulting in neurite outgrowth.

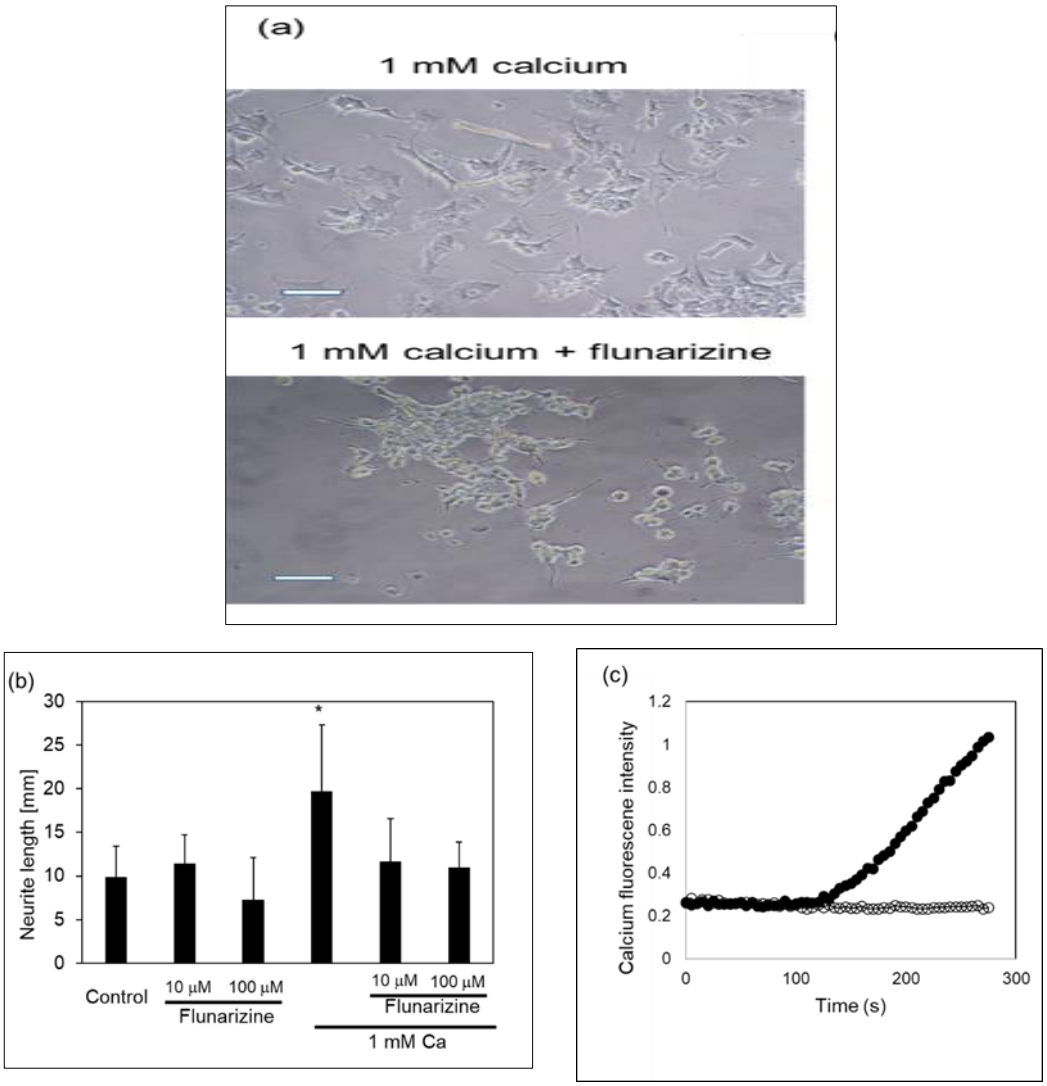


Figure 5 Effect of calcium channel blocker flunarizine on calcium-induced neurite outgrowth and intracellular calcium. (a) PC12 HS cells were treated with 1 mM calcium in the presence or absence of 10 μM or 100 μM flunarizine. After 72 h, photographs were taken and (b) neurite length was measured. Scale bars show 500μm. Data are expressed as the mean ± SD. Statistical significance was determined by ANOVA: *p≤0.05 relative to control. (c) PC12 HS cells were treated with mM CaCl2 in the presence (closed circles) or absence of 100 mM flunarizine (open circles). The change in fluorescent intensity was measured every 10 s under confocal microscopy, and the relative fluorescent intensity was plotted.

3.4. Elevated concentration of extracellular calcium induces phosphorylation of Akt and CREB.

Several studies have suggested that phosphorylation of intracellular signal-transducing proteins ERK, Akt, or the transcription factor CREB is involved in neurite extension [13,14]. To investigate whether elevated extracellular calcium can activate ERK, Akt, or CREB, PC12 HS cells were treated with 1 mM $CaCl_2$ for 60 min, and cell lysates were immunoblotted. Elevated extracellular calcium levels increased the phosphorylation of Akt and CREB, but not ERK (Figure 6). Phosphorylation of Akt and CREB increased by approximately 4-fold and 2.5-fold, respectively, suggesting that elevated extracellular calcium activates the Akt/CREB pathway. This result was also supported by the increase in the mRNA expression of BDNF and PDGF, which are regulated by the activation of CREB.

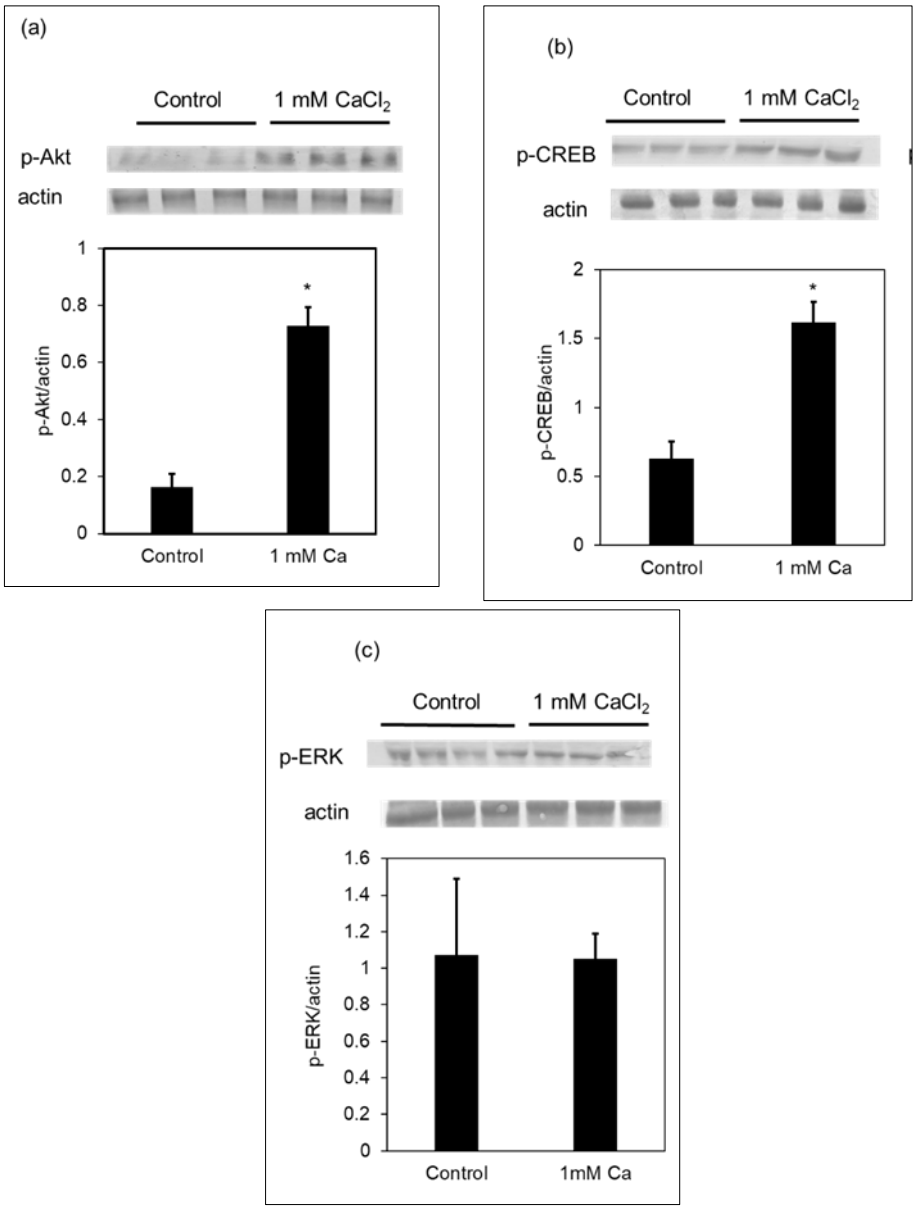


Figure 6 Effect of extracellular calcium on phosphorylation of AKt, ERK, and CREB. PC12 HS cells were treated with 1 mM calcium or vehicle. After 60 min, cells were collected and the phosphorylation levels of AKt, and CREB were determined, and the expression was normalized with β-actin. Statistical significance was determined by ANOVA: *p≤0.05 relative to control.

There is a close connection between oxidative stress and intracellular calcium homeostasis. Overload of intracellular calcium causes oxidative stress, leading to the increase of antioxidant enzymes. To eliminate the possibility that abnormal calcium homeostasis promotes neurite outgrowth, we investigated whether elevated extracellular calcium increases the expression of antioxidant enzymes. The expression of antioxidant enzymes did not change with the addition of 1 mM extracellular calcium, suggesting that neurite extension was not due to an overload of intracellular calcium.

4. Discussion

We showed that elevated extracellular calcium promotes neurite outgrowth in PC12 HS cells via the CaSR. Koike showed that elevated extracellular calcium did not promote neurite outgrowth in PC12 cells [6]. Activation of the endogenous CaSR in SCG neurons did not enhance neurite outgrowth in the absence of NGF, but SCG neurons overexpressing CaSR induced neurite outgrowth via extracellular calcium [4, 5]. These results suggest that PC12 HS cells express a higher amount of CaSRs than PC12 and SCG neurons.

The CaSR is a member of the G protein-coupled receptor superfamily. The CaSR controls multiple signaling pathways. In proximal tubular OK cells expressing CaSR, elevated extracellular calcium elicited phosphorylation of ERK1/2 and increased intracellular calcium [15]. In osteoclast precursor cells (RAW 264.7), elevated extracellular calcium levels activate the phosphoinositide 3-kinase/Akt pathway and induce migration [16]. Vizart et al. reported that activation of CaSR leads to activation of ERK1/2 in CaSR-overexpressing SCG neurons [5]. In PC12 HS cells, activation of CaSR activated Akt, but not ERK, leading to neurite outgrowth. Neurite outgrowth due to elevated extracellular calcium levels may be induced by different pathways in SCG neurons and PC12 HS cells.

CaSR-mediated activation leads to activation of phospholipase β, resulting in production of inositol 1,4,5-triphosphate (IP_3), which mobilizes cytosolic Ca^{2+} from intracellular Ca^{2+} stores and diacylglycerol (DAG), which activates receptor-operated Ca^{2+} channels (TRPC6) in the plasma membrane [17, 18]. In this study, an increase in intracellular calcium was suppressed by flunarizine, which blocks not only T-type calcium channels but also high voltage-activated calcium channels (L, N, P, Q, and R types) and receptor-operated Ca^{2+} influx [19]. This result suggests that elevated extracellular calcium may increase intracellular calcium levels via TRC6 in PC12HS cells, leading to neurite outgrowth.

5. Conclusion

In this study, we showed that elevated extracellular calcium can induce the phosphorylation of Akt and CREB, and an increase in intracellular calcium through the calcium-sensing receptor, leading to the promotion of neurites. PC12 HS cells are a useful model to investigate the regulation of extracellular calcium via calcium-sensing receptors in neuronal cells.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare no competing interests.

Author Contributions

Concept, design, and supervision by Y. H.; data collection and analysis by S. T; Literature search and manuscript writing: Y. H

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