Biochemical and Biophysical Research Communications 379 (2009) 11–15

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Developmental changes in the regulation of calcium-dependent neurite outgrowth

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ARTICLE INFO

Article history:
Received 14 November 2008
Available online 7 December 2008

Keywords:
Developmental changes
Neurite outgrowth
Calcium
IP3 receptor
Ryanodine receptor
DRG
Sensory neuron

ABSTRACT

Intracellular calcium ions (Ca^{2+}) have an essential role in the regulation of neurite outgrowth, but how outgrowth is controlled remains largely unknown. In this study, we examined how the mechanisms of neurite outgrowth change during development in chick and mouse dorsal root ganglion neurons. 2APB, a potent inhibitor of inositol 1,4,5-trisphosphate (IP_3) receptors (IP_3R), inhibited neurite outgrowth at early developmental stages, but not at later stages. In contrast, pharmacological inhibition with Ni^{2+}, Cd^{2+}, or dantrolene revealed that ryanodine receptor (RyR)-mediated Ca^{2+}-induced Ca^{2+} release (CICR) was involved in neurite outgrowth at later stage, but not at early stages. The distribution of IP_3R and RyR in growth cones also changed during development. Furthermore, pharmacological inhibition of the Ca^{2+}–calmodulin-dependent phosphatase calcineurin with FK506 reduced neurite outgrowth only at early stages. These data suggest that the calcium signaling that regulates neurite outgrowth may change during development from an IP_3R-mediated pathway to a RyR-mediated pathway.

It has been well-established that intracellular free calcium ions (Ca^{2+}) act as important second messengers that regulate signal transduction processes that control nerve growth, including neurite outgrowth and axonal pathfinding (for a review, see [1,2]). Accumulating evidence has shown that Ca^{2+} elevation in growth cones is required for cellular responses to many guidance cues (for a review, see [1,3]) such as nerve growth factor [4], brain-derived neurotrophic factor [5], netrin-1 [6,7], and myelin-associated glycoprotein [8,9]. However, the molecular mechanisms of Ca^{2+}-dependent regulation of neurite outgrowth still remain incompletely understood.

It has been reported that different signaling pathways mediate regenerative and developmental axon growth [10], suggesting that the signaling that regulates neurite outgrowth changes from the developing organism to the adult. Developing immature neurons do not respond to the myelin-associated neurite growth inhibitor NI-35/250 with a small transient Ca^{2+} rise, but adult mature neurons respond to NI-35/250 with drastic growth cone collapse and a large elevation in Ca^{2+} [11]. This observation suggests that a developmental change in response to the CNS myelin-associated neurite growth inhibitor occurs. As a step in elucidating the molecular mechanisms of regulation of neurite outgrowth, we tested whether Ca^{2+}-dependent regulation of neurite outgrowth changes during development. We report developmental changes in Ca^{2+}-dependent regulation of neurite outgrowth in chick and mouse sensory neurons.

Materials and methods

Explant primary cultures of chick and mouse DRG neurons. Primary dissociated cell cultures of chick and mouse dorsal root ganglion (DRG) neurons were performed as previously reported [12]. Briefly, DRG neurons from embryonic day (ED) 8, 10, and 15 chick embryos, ED16 mouse embryos, or postnatal day (PD) 1 and 6 mice were dissected and directly plated onto poly-L-lysine- and laminin (Invitrogen)-coated glass-bottom dishes (Iwaki) in Leibovitz-15 medium (Invitrogen) containing nerve growth factor (NGF) (10 ng/mL, Wako), gentamycin (Sigma), 0.6% glucose, 2 mM glutamine, and 10% fetal bovine serum (Invitrogen). The explants were cultured at 37 °C for 24 h, by which time most neurons had produced neurites.

Antibodies. Monoclonal rat antibodies against type 1 IP_3R (IP_3R1) (4C11 monoclonal antibody) were produced as previously described [13]. Mouse anti-ryanodine receptor (RyR) antibody (Sig-

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doi:10.1016/j.bbrc.2008.11.128

ma), mouse anti-PP2B-A (calcineurin) antibody (Santa Cruz Biotech), and mouse anti-CaMKIIα antibody (Santa Cruz Biotech) were commercially obtained. Alexa488-labeled goat anti-mouse IgG (Molecular Probes), TRITC-labeled rat IgG (Sigma), and non-immune (non-specific) mouse IgG (Calbiochem) were also commercially obtained.

Immunocytochemistry. Chick DRG cultures from ED8 and ED15 embryos were incubated at 37 °C for 4 h. The cells were fixed in warm 4% paraformaldehyde in culture medium at 37 °C for 1 h. After rinsing with phosphate buffered-saline (PBS), the cells were permeabilized with 0.3% Triton X-100 (Sigma) in PBS for 2 min, blocked with 1% bovine serum albumin (Sigma) in 0.1% Triton X-100 for 1 h, probed with primary antibodies against IP_3R1 (4C11 monoclonal antibody, 1 μg/ml) and RyR (1:200, Sigma) overnight at 4 °C, and then incubated with fluorescence-conjugated secondary antibodies for 1 h at room temperature. The cells were observed by epifluorescence using an Olympus IX-71 inverted microscope (Olympus). In all experiments, matched control immunostainings were carried out with non-immune IgG or without primary antibody.

Pharmacological treatment and neurite outgrowth assay. Chick and mouse DRG cultures were incubated at 37 °C for 24 h. The cells were exposed to pharmacological reagents at the time of plating and incubated at 37 °C for 24 h. 2-Aminoethoxydiphenyl borate (2APB, Calbiochem) at 100 μM, dantrolene (DTL, Sigma) at 1 μM, nickel chloride hexahydrate (Ni, Wako) at 150 μM, cobalt chloride (Wako) at 20 μM, FK506 (Tacrolimus, LC Lab) at 1 or 5 μM, and KN93 (Calbiochem) at 10 μM were dissolved in culture medium. Rapamycin (Calbiochem) at 1 μM and KN92 (Calbiochem) at 10 μM were used for the control analogs of FK506 and KN93, respectively. The cells were then fixed in pre-warmed 4% paraformaldehyde in culture medium at 37 °C for 1 h. Phase-contrast images were captured with a cooled CCD camera (CoolSnap-HQ, Photometrics). Neurite length was measured as the distance from the edge of the explants to the center of the growth cone in a crow line and analyzed with Metamorph software (Molecular Device).

Results and discussion

Changes in calcium source required for neurite outgrowth in chick DRG neurons

The concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) has long been known to be an important factor in the regulation of neurite outgrowth. An optimal range of $[Ca^{2+}]_i$ in growth cones is required for proper neurite outgrowth ([14], for a review, see [1]). Therefore, the amplitude of the $[Ca^{2+}]_i$ is considered to be an important factor in calcium signaling. However, Ooashi et al. reported that calcium-induced calcium release (CICR) mediated by type 3 ryanodine receptors (RyR) in the growth cone led to an attraction response, whereas repulsion was seen in the absence of RyR function [15], suggesting that the source of Ca^{2+} leading to a change in $[Ca^{2+}]_i$ is more important determinant of the growth cone turning response. Therefore, we examined whether the source of Ca^{2+} required for neurite outgrowth changes during development.

Calcium release from internal stores through inositol 1,4,5-trisphosphate receptors (IP_3R) in growth cones is reported to be an essential event in Ca^{2+}-dependent neurite outgrowth in chick DRG neurons from ED 10 embryos [12]. We first examined whether IP_3-induced calcium release (IICR) is an important event in the intracellular signaling pathway regulating neurite outgrowth during development. As expected, the potent IP_3R blocker 2APB significantly inhibited neurite outgrowth in chick DRG neurons from ED8 and 10 embryos (Fig. 1A and B). However, no obvious effect of 2APB on neurite outgrowth was seen in chick DRG neurons from ED15 embryos (Fig. 1A and B). In contrast, the RyR inhibitor dantrolene (DTL) did not affect neurite outgrowth in chick DRG neurons from ED8 and 10 embryos, whereas DTL significantly inhibited outgrowth at ED15 (Fig. 1A). A high dose of ryanodine (100 μM), which is well-known to inhibit RyR function, also showed a similar inhibitory effect on neurite outgrowth at ED15, but not at ED8 and 10 (data not shown). Furthermore, nickel chloride (Ni), an inhibitor of T- and R-type voltage-dependent calcium channels (VDCC), reduced neurite outgrowth at ED15, but not at ED8 and 10 (Fig. 1A). To confirm the effect of VDCC inhibition, we also used a global VDCC inhibitor, cobalt chloride to inhibit N, P/Q, and L-type VDCC, and obtained similar results (data not shown). These findings suggest that both calcium release from internal stores through RyR, and calcium influx through voltage-dependent calcium channels, have an important role in neurite outgrowth in chick DRG neurons from ED15 embryos. Thus, CICR, which is well-known to elevate $[Ca^{2+}]_i$ by Ca^{2+}-induced RyR activation, may be important for neurite outgrowth in chick DRG neurons at later stages of development. Thus, our data suggest that changes in the calcium source from IICR to CICR are required for neurite outgrowth in chick DRG neurons during development.

The type 1 IP_3R (IP_3R1), a major neuronal member of the IP_3R family, is expressed in the growth cone of chick DRG neurons [12]. Immunocytochemistry revealed that IP_3R1 was primarily detected in the central domain of the growth cone and along the neurite of chick DRG neurons from ED8 embryos (Fig. 1C), consistent with a previous report [12]. In contrast, RyR was faintly distributed in the growth cone, including the filopodia, and distinct localization was not seen in the growth cones of ED8 chick DRG neurons (Fig. 1C). Conversely, at a later stage (ED15), IP_3R1 was distributed in the entire region of the lamellipodia, and the cluster-like distribution of IP_3R1 in the central domain of the growth cone was not clearly observed (Fig. 1C), while RyR was primary detected in filopodia and the leading edge of the lamellipodia of the growth cone (Fig. 1C). Thus, the distribution of IP_3R1 and RyR in growth cones at a later stage (ED15) showed a different distribution pattern from that seen at an earlier stage (ED8). These pharmacological (Fig. 1A) and immunocytochemical (Fig. 1C) data suggest that IICR-mediated localized calcium signaling in the central domain of the growth cone may be important in neurite outgrowth at early stages, and at later stages, the important calcium signaling in neurite outgrowth may switch to CICR through RyR in other subregions of the growth cone, such as filopodia and the leading edge of lamellipodia.

The calcineurin inhibitor FK506 inhibits neurite outgrowth only at early stages in chick DRG neurons

As a step in understanding the molecular events downstream of a calcium rise, we next examined the functional involvement of a Ca^{2+}- and calmodulin (CaM)-dependent protein phosphatase, calcineurin (CaN), and a Ca^{2+}–CaM-dependent kinase (CaMK). To address this issue, we characterized the pharmacological effects of the CaN inhibitor FK506, also known as immunosuppressant, and the CaMK inhibitor KN93, which blocks both CaMKII and CaMKIV, on neurite outgrowth in chick DRG neurons from ED8, 10, and 15 embryos. Pharmacological treatment with FK506 for 24 h significantly inhibited neurite outgrowth at ED8 and 10, while no significant effect of FK506 was found in neurite outgrowth at ED15 (Fig. 2A and B). Treatment with 1 μM rapamycin, a control analog of FK506 that is a ligand of the FK506-binding protein but not CaN, had no effect (data not shown), suggesting that the inhibitory effect of FK506 on neurite outgrowth was due to specific blockade of CaN function. These findings suggest that CaN may promote neurite outgrowth at early stages, but may not participate in the promotion of neurite outgrowth at later stages in chick DRG neurons. Pharmacological inhibition of CaN has been reported to inhi-

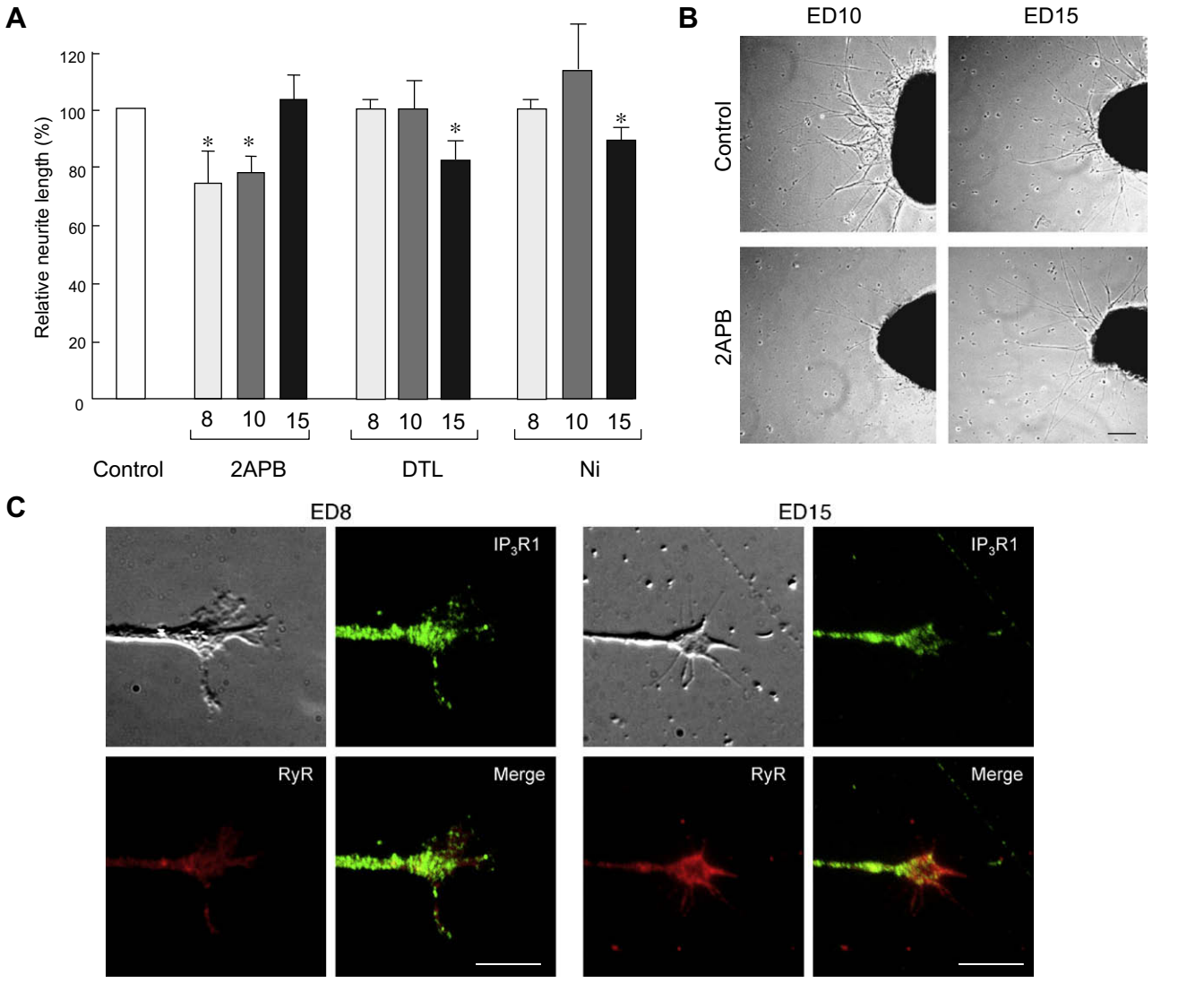

Fig. 1. Developmental changes in the calcium source required for neurite outgrowth in chick DRG neurons. (A) Neurite outgrowth assay with pharmacological reagents in chick DRG explant cultures from ED8, 10, and 15 chick embryos. Pharmacological reagents were added to the culture medium at the time of plating, and the DRG explants were exposed to the agent for 24 h. The data shown are the relative averages (%) where 100% is the neurite length in the control ± standard error of the mean (SEM). Stars (*) indicate significantly lower outgrowth than with control reagents (vehicle) ($n > 5$); $p < 0.001$ according to Student's unpaired t-test. 2APB: 2-aminoethoxydiphenyl borate (100 μM) used to inhibit IP_3R (blockade of IP_3-induced Ca^{2+} release from internal stores), DTL: dantrolene (1 μM) used to inhibit RyR (blockade of Ca^{2+} release from internal stores through RyR), Ni: nickel chloride hexahydrate (150 μM) used to inhibit VDCC. Data show that 2-APB significantly decreased the neurite length. (B) Photomicrographs of chick DRG explant cultures from ED10 and ED15 embryos with control vehicle reagent (Control) and 2APB. Scale bar indicates 100 μm. (C) Distribution of IP_3R1 and RyR in the growth cone. Chick DRG neurons from ED8 and ED15 embryos cultured on laminin were immunostained with anti-IP_3R1 and anti-RyR antibodies. Control immunostaining experiments showed absence of staining (data not shown). Scale bar indicates 10 μm.

bit [16–19], or promote [20,21] neurite outgrowth, depending on the culture system and cell type used. We found a developmental change in the pharmacological inhibition of neurite outgrowth in the same culture system and cell type, but we did not observe inhibition of neurite outgrowth by CaN at later stages.

Next, we characterized the effects of the CaMK inhibitor KN93 on neurite outgrowth. Chick DRG neurons exposed to KN93 showed no significant alteration in neurite outgrowth (Fig. 2A and B), although KN93 may increase neurite outgrowth slightly as development proceeds. Since KN93 inhibits not only CaMKII, but also CaMKI and CaMKIV, the data suggest that most CaMK is not involved in promotion of neurite outgrowth in the developmental stages of chick DRG neurons. Previous studies examining a role for CaMK in neurite outgrowth showed conflicting results that varied depending on the cell type and animal species examined. Expression of active CaMKII promotes neurite outgrowth in neuroblastoma cells [22,23], but inhibits outgrowth in PC12 cells [24] and Xenopus retinotectal neurons [25]. A recent study demonstrates that CaMKI, but not CaMKII, positively regulates axonal extension in neonatal hippocampal and postnatal cerebellar granule neurons [26]. In DRG neurons from ED8 and 15 embryos, we did not detect CaMKIIα and CaMKIIγ immunocytochemically (data not shown). Therefore, we suggest that CaMKII is unlikely to function in chick DRG neurons, but we have not yet examined the expression of CaMKI in these cells.

Changes in calcium–calmodulin-dependent regulation of neurite outgrowth in mouse DRG neurons

We next examined whether the developmental changes seen in chick DRG neurons can be observed in DRG neurons from other animal species. The same pharmacological experiments were performed in DRG neurons from ED16 mouse embryos, and postnatal day (PD) 1 and PD6 mice. 2APB significantly inhibited neurite out-

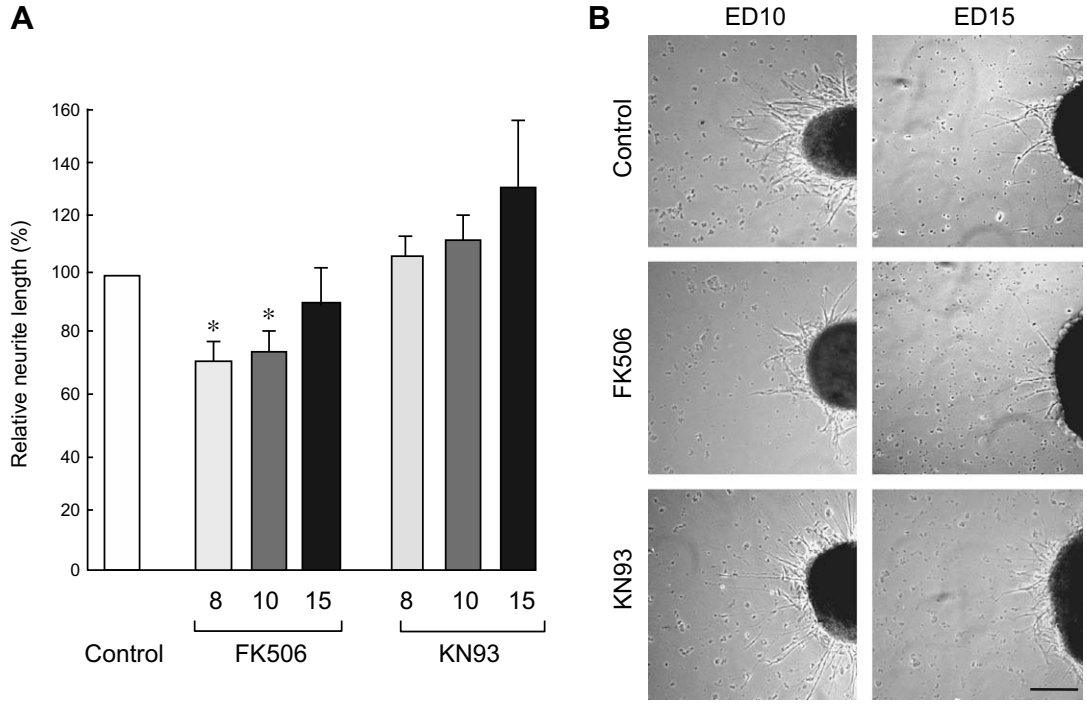

Fig. 2. Developmental changes in the role of CaN in neurite outgrowth of chick DRG neurons. (A) Neurite outgrowth assay with pharmacological reagents in chick DRG explant cultures from ED8, 10, and 15 embryos. Pharmacological reagents were added to the culture medium at the time of plating, and the DRG explants were exposed to the agent for 24 h. The data shown are the relative averages (%) where 100% is the neurite length in the control ± standard error of the mean (SEM). Note that the absolute length of the neurite and the number of neurites in a cell mass at ED15 in the culture was decreased to about 60% of those at ED8 (data not shown). Stars (*) indicate significantly lower outgrowth than with control reagents (vehicle) ($n > 5$); $p < 0.001$ according to Student's unpaired t-test. FK506 (5 μM) and KN93 (10 μM) were used to inhibit CaN and CaMK, respectively. (B) Photomicrographs of chick DRG explant cultures from ED10 and ED15 embryos with control vehicle reagent (Control), FK506 and KN93. Scale bar indicates100 μm.

growth of DRG neurons from ED16 mouse embryos and PD1 newborn mice, but not from PD6 postnatal mice (Fig. 3A). Similarly, FK506 inhibited neurite outgrowth in DRG neurons from ED16 embryos and PD1 newborn mice, but not from PD6 postnatal mice (Fig. 3A). These developmental changes in the calcium source and the role of CaN that contributed to neurite outgrowth of mouse DRG neurons were similar to what was observed in chick DRG neurons. In contrast, the CaMKI, CaMKII, and CaMKIV inhibitor KN62, which did not affect neurite outgrowth of mouse DRG neurons at ED16 and PD1, inhibited neurite outgrowth of mouse DRG neurons at PD6 (Fig. 3A). These data suggest that CaN has an important role in neurite outgrowth, using 2APB-sensitive IICR at early stages

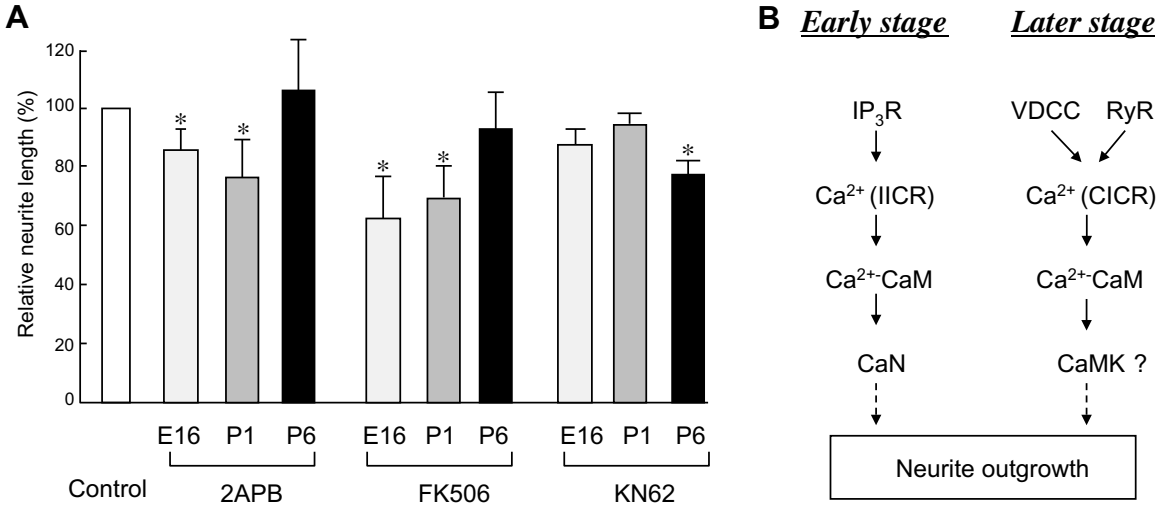

Fig. 3. Developmental changes in the role of CaN and CaMK in neurite outgrowth of mouse DRG neurons. (A) Neurite outgrowth assay with pharmacological reagents in mouse DRG explant cultures from ED16 embryos, PD1 newborn mice, and PD6 mice. Pharmacological reagents were added to the culture medium at the time of plating, and the DRG explants were exposed to the agent for 24 h. The data shown are the relative averages (%) where 100% is the neurite length in the control ± standard error of the mean (SEM). Stars (*) indicate significantly lower outgrowth than with control reagents (vehicle) ($n > 5$); $p < 0.001$ according to Student's unpaired t-test. FK506 (5 μM) and KN62 (10 μM) were used to inhibit CaN and CaMK, respectively. (B) Schematic drawing represents developmental changes in calcium-dependent regulation of neurite outgrowth. Note the utilization of IICR-CaN at early stages and CICR-CaMK at later stages.

(ED16 and PD1) and CaMK at later stages of neurite outgrowth (PD6).

Taken together, the data in this study using pharmacological inhibition of neurite outgrowth of chick and mouse DRG neurons raise the possibility that as development progresses, the calcium-dependent regulation of neurite outgrowth may change to a different mode: the key players for neurite outgrowth at early stages involve IICR-activated CaN, and CICR-activated CaMK may occur at later stages (Fig. 3B). Interestingly, Wen et al. proposed a switch-like mechanism mediated by CaMKII and CaN that controls the direction of Ca^{2+}-dependent growth cone turning behavior [27]. They reported that a difference in the level of $[Ca^{2+}]_i$ elevation caused these enzymes to activate differently: a high level of $[Ca^{2+}]_i$ elevation activated CaMKII, while a low level of $[Ca^{2+}]_i$ elevation activated CaN [27]. We hypothesize that the main Ca^{2+} source in growth cones that promotes neurite outgrowth may change during development and give rise to changes in the $[Ca^{2+}]_i$ elevation level, leading to activation of different signaling cascades. Finally, we also speculate that neuronal development and regeneration may be regulated by different Ca^{2+}-dependent neurite outgrowth mechanisms.

Acknowledgments

This work was supported by a Grant-in-Aid from the Ministry of Education, Culture, Sport, Science, and Technology of Japan (to K.T.). We are grateful to Ms. Junko Yamaoka at Yokohama City University and Ms. Naoko Tokushige at Brain Science Institute of RIKEN for experimental assistance.

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