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ERK signaling mediates CaSR-promoted axon growth

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HIGHLIGHTS

- We have investigated how CaSR activation enhances sympathetic axon growth.
- CaSR activation promotes phosphorylation of ERK1 and ERK2.
- Inhibition of ERK1/ERK2 phosphorylation blocks CaSR-promoted axon growth.
- CaSR-promoted axon growth requires a discrete region of the cytoplasmic domain.

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ABSTRACT

The extracellular calcium-sensing receptor (CaSR) is a G-protein coupled receptor that monitors the systemic extracellular free ionized calcium level ($[Ca^{2+}]_o$) in organs involved in systemic $[Ca^{2+}]_o$ homeostasis. CaSR is widely expressed in the nervous system and its activation promotes axon and dendrite growth during development, but the mechanism by which it does this is not known. Here we show that enhanced axon growth and branching from cultured embryonic sympathetic neurons by activation of the endogenous CaSR depends on the presence of nerve growth factor (NGF). Our observation that activation of overexpressed CaSR promotes axon growth in NGF-free medium has enabled us to investigate CaSR downstream signaling contributing to axon growth in the absence of NGF signaling. We show that activation of overexpressed CaSR leads to activation of ERK1 and ERK2, and pharmacological inhibition of CaSR-dependent ERK1/ERK2 activation prevents CaSR-dependent axon growth. Analysis of axon growth from cultured neurons expressing deletion mutants of the CaSR cytoplasmic tail revealed that the region between alanine 877 and glycine 907 is required for promoting axon growth that is distinct from the high-affinity filamin-A binding site that has previously been implicated in ERK1/ERK2 activation.

1. Introduction

The CaSR plays a crucial role in monitoring and maintaining $[Ca^{2+}]_o$ within very narrow physiological limits and is conspicuously expressed in the tissues and organs involved in systemic calcium homeostasis [1]. The CaSR is also widely expressed in the peripheral and central nervous system, where it has been implicated in a diversity of functions [2]. These include regulating axon and dendrite growth [3], the migration and/or maintenance of hypothalamic GnRH neurons [4] and the regulation of neuronal excitability and synaptic transmission [5,6].

The molecular mechanisms by which CaSR exerts its effects on neurons are poorly understood. The CaSR is a member of the C family of G-protein coupled receptors that associates with three main heterotrimeric G protein complexes, $G_{q/11}$, $G_{i/o}$ and $G_{12/13}$, and thereby modulates the activity of a wide variety of downstream signaling networks, including PLC-mediated Ca^{2+} mobilization, cAMP, Rho kinase and the MAP kinases ERK1/2, p38 and JNK [7]. The aim of this study was to ascertain how CaSR activation influences axon growth and branching using the well-characterized, experimentally tractable sympathetic neurons of the mouse superior cervical ganglion (SCG) [8]. Previous work has shown that expression of the CaSR peaks in these neurons in the immediate perinatal period and that activating the CaSR during this stage of development enhances NGF-promoted axon growth, and that this is important for the establishment of the appropriate level of sympathetic innervation *in vivo* [3]. Our demonstration that activation of overexpressed CaSR enhances axon growth in the absence of NGF has enabled us to investigate how the CaSR influences axon growth

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without the complication of concomitant NGF signaling. We show that CaSR-promoted ERK activation contributes to CaSR-promoted axon growth and identify the region of the CaSR C-terminal domain required for axon growth.

2. Materials and methods

2.1. Neuron cultures

Dissociated cultures of SCG neurons from CD-1 mice were grown on poly-ornithine/laminin coated 35 mm tissue culture dishes (Greiner) in Hams F14 medium [9] with 0.25% Albumax I (Invitrogen). Survival was estimated as described [9]. The neurite arbors of non-transfected neurons were labelled with calcein-AM (1:1000, Invitrogen). Neurons transfected with plasmids encoding full-length CaSR or CaSR mutants were co-transfected with a YFP plasmid. Fast-Sholl analysis was carried out on imaged neurons [10].

2.2. Plasmids

The pFLCaSR plasmid was generated by cloning the open reading frame of human CaSR into pcDNA3.1. The pG907stopCaSR and pA877stopCaSR plasmids were generated by site directed mutagenesis. Transfection was carried out using the Neon Transfection system (Invitrogen).

2.3. Immunocytochemistry

Cultures were fixed in ice-cold methanol for 10 min, washed in PBS, blocked and permeabilized with 5% BSA with 0.02% Triton-X100 in PBS. The cells were incubated with primary antibody in 1% BSA at 4 °C for 18 h. The primary antibodies were: anti-βIII tubulin (Promega, 1:1000), anti-CaSR to the CaSR N-terminal sequence (Imgenex, 1:1000), anti-phospho ERK1/2 and anti-total ERK1/2 (Cell Signaling Technology, 1:100). After washing, the cells were incubated with appropriate secondary antibodies conjugated to either Alexa-488 or Alexa-546 (Invitrogen), 1:600 for 90 min. Staining intensity was quantified using pixel intensity using the Volocity software (PerkinElmer).

3. Results

3.1. CaSR-promoted neurite growth is NGF-dependent

Previous work has shown that activating the CaSR in cultured SCG neurons with elevated levels of $[Ca^{2+}]_o$ enhances NGF-promoted axon growth in the immediate perinatal period [3]. To ascertain whether or not CaSR activation is able to enhance neurite growth independently of NGF, we compared neurite growth from E18 SCG neurons cultured with and without NGF in media containing 2.3 mM (maximally-activating) and 0.7 mM (minimally-activating) levels of $[Ca^{2+}]_o$ [3]. Because E18 SCG neurons are dependent on NGF for survival, we added a broad-spectrum caspase inhibitor (Boc-D-FMK) to the medium to prevent apoptosis.

In accordance with published observations [3], the neurite arbors of NGF-supplemented neurons grown in medium containing 2.3 mM $[Ca^{2+}]_o$ were much larger and more branched than those of neurons grown with 0.7 mM $[Ca^{2+}]_o$. There were highly significant differences in neurite length (Fig. 1A) and branch point number (Fig. 1B), and the Sholl profiles displayed clear differences in NGF-supplemented cultures (Fig. 1C). In contrast, the size and complexity of the neurite arbors of neurons grown without NGF were not significantly different in media containing 0.7 mM and 2.3 mM $[Ca^{2+}]_o$ (Figs. 1A–C). These findings suggest that activation of the endogenous CaSR is insufficient to enhance the low level of neurite growth that occurs in the absence of NGF, but enhances the magnitude of NGF-promoted neurite growth.

3.2. Over-expression of CaSR promotes neurite growth in the absence of NGF

The requirement for NGF in CaSR-promoted neurite growth complicates investigation of the signaling pathways downstream of CaSR that mediate this effect. Because enhancement of neurite growth by CaSR activation is only observed in SCG neurons at the developmental peak of CaSR expression [3], we tested whether overexpression of CaSR would enhance neurite growth in the absence of NGF. Robust high-level CaSR expression was achieved by transfecting neurons with a pcDNA3.1 vector containing full-length CaSR (pFLCaSR). Quantification of CaSR immunofluorescence confirmed increased CaSR expression in neurons transfected with pFLCaSR (Fig. 2A).

In the absence of NGF, the neurite arbors of E18 SCG neurons transfected with pFLCaSR were significantly larger than those of control transfected neurons in medium containing 2.3 mM $[Ca^{2+}]_o$ (Fig. 2B, and C). There was no significant difference in neurite arbor size between pFLCaSR transfected and control transfected neurons in medium containing 0.7 mM $[Ca^{2+}]_o$. All cultures were supplemented with Boc-D-FMK to prevent neuronal death in the absence of NGF. These findings suggest that overexpression of CaSR enhances neurite growth in NGF-free medium containing activating levels of $[Ca^{2+}]_o$.

Given the above results, we explored the possibility that upregulation of CaSR expression contributes to enhanced neurite growth from non-transfected neurons grown with NGF and activating levels of $[Ca^{2+}]_o$. We cultured E18 SCG neurons with and without NGF in media containing either 0.7 or 2.3 mM $[Ca^{2+}]_o$ and estimated the relative levels of CaSR immunofluorescence after 24 h. All cultures received Boc-D-FMK to prevent apoptosis. There was no significant difference in CaSR immunofluorescence in neurons cultured with either 0.7 or 2.3 mM $[Ca^{2+}]_o$ in the absence of NGF (Fig. 2D). However, in the presence of NGF, the level of CaSR immunofluorescence was over two-fold higher in neurons cultured in medium containing 2.3 mM $[Ca^{2+}]_o$ than in neurons cultured in medium containing 0.7 mM $[Ca^{2+}]_o$ (Fig. 2D). Images of CaSR-labelled neurons cultured with NGF in medium containing either 0.7 mM or 2.3 mM $[Ca^{2+}]_o$ are illustrated in Fig. 2E. This suggests that CaSR expression is upregulated in medium containing NGF and activating levels of $[Ca^{2+}]_o$ and that this in turn contributes to the enhanced neurite growth observed from late fetal neurons cultured under these conditions.

3.3. ERK1/ERK2 activation by CaSR over-expression contributes to neurite growth

To elucidate the molecular mechanism underlying the enhancement of neurite growth from SCG neurons by activated CaSR, we explored a common link in intracellular signaling between CaSR signaling and neurite growth. ERK1 and ERK2 are activated by the CaSR in parathyroid cells, fibroblasts and kidney cell lines [11–15] and NGF-promoted ERK1/ERK2 activation in PC12 cells and SCG neurons contributes to the neurite growth response [16–20].

To investigate whether ERK1/ERK2 signaling contributes to CaSR-promoted neurite growth from E18 SCG neurons, we used immunofluorescence to estimate the relative levels of phospho-ERK1/ERK2 in neurons. Phospho-ERK1/ERK2 immunofluorescence was clearly elevated in NGF-supplemented neurons grown in 2.3 mM $[Ca^{2+}]_o$ medium compared with NGF-supplemented neurons grown in 0.7 mM $[Ca^{2+}]_o$ medium and neurons grown in NGF-free medium containing either 0.7 mM or 2.3 mM $[Ca^{2+}]_o$