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Mechanisms controlling neurite outgrowth in a pheochromocytoma cell line: The role of TRPC channels

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Abstract

Transient Receptor Potential Canonical (TRPC) channels are implicated in modulating neurite outgrowth. The expression pattern of TRPC changes significantly during brain development, suggesting that fine-tuning TRPC expression may be important for orchestrating neuritogenesis. To study how alterations in the TRPC expression pattern affect neurite outgrowth, we used nerve growth factor (NGF)-differentiated rat pheochromocytoma 12 (PC12) cells, a model system for neuritogenesis. In PC12 cells, NGF markedly up-regulated TRPC1 and TRPC6 expression, but down-regulated TRPC5 expression while promoting neurite outgrowth. Overexpression of TRPC1 augmented, whereas TRPC5 overexpression decelerated NGF-induced neurite outgrowth. Conversely, shRNA-mediated knockdown of TRPC1 decreased, whereas shRNA-mediated knockdown of TRPC5 increased NGF-induced neurite extension. Endogenous TRPC1 attenuated the anti-neuritogenic effect of overexpressed TRPC5 in part by forming the heteromeric TRPC1–TRPC5 channels. Previous reports suggested that TRPC6 may facilitate neurite outgrowth. However, we found that TRPC6 overexpression slowed down neuritogenesis, whereas dominant negative TRPC6 (DN-TRPC6) facilitated neurite outgrowth in NGF-differentiated PC12 cells. Consistent with these findings, hyperforin, a neurite outgrowth promoting factor, decreased TRPC6 expression in NGF-differentiated PC12 cells. Using pharmacological and molecular biological approaches, we determined that NGF up-regulated TRPC1 and TRPC6 expression via a p75^{NTR}-IKK₂-dependent pathway that did not involve TrkA receptor signaling in PC12 cells. Similarly, NGF up-regulated TRPC1 and TRPC6 via an IKK₂ dependent pathway in primary cultured hippocampal neurons. Thus, our data suggest that a balance of TRPC1, TRPC5, and TRPC6 expression determines neurite extension rate in neural cells, with TRPC6 emerging as an NGF-dependent “molecular damper” maintaining a submaximal velocity of neurite extension.

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Keywords

TRPC channels; NF- κ B; IKK $_2$; neurite outgrowth; PC12 cells; hippocampal neurons

INTRODUCTION

Neurite outgrowth is a crucial step during neuronal differentiation. Multiple signaling pathways regulate neurite formation and extension. For instance, accumulating evidence suggests that the activation of PI3K (Kobayashi et al, 1997), Akt (Read and Gorman, 2009), c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (Takeda et al, 2000), NOS/nitric oxide (Tojima et al, 2009), and the inhibitor κ B kinase 2 (IKK $_2$)/NF- κ B signaling cascades (Azoitei et al, 2005) promote neurite outgrowth. Interestingly, some regulatory proteins modulate neurite outgrowth in an isoform specific manner. For instance, Ca $^{2+}$ -calmodulin-dependent protein kinase IV (Tai et al, 2008) and I γ (Davare et al, 2009) promote dendrite formation and extension, whereas Ca $^{2+}$ -calmodulin-dependent protein kinase II limits dendrite outgrowth and promotes the maturation of synapses (Wu and Cline, 1998). Consistent with these distinct effects of different CaMK isoforms, Ca $^{2+}$ influx has been reported to either stimulate or inhibit neurite outgrowth in various neuronal model systems (for review see (Gomez and Zheng, 2006)).

Transient Receptor Potential Canonical (TRPC) proteins form Ca $^{2+}$ permeable channels in the plasma membrane of various cell types, including neuronal and neuroendocrine cells. The TRPC subfamily consists of seven members (TRPC1 – TRPC7, (Clapham, 2003)). Four identical or homologous subunits assemble together to form a functional channel (Ramsey et al, 2006). Importantly, the biophysical properties of heterotetrameric TRPCs may differ from those of homotetrameric TRPCs. For instance, TRPC1 and TRPC5 form a heterotetrameric channel with markedly reduced cation permeability as compared with homotetrameric TRPC5 channels (Strubing et al, 2001).

In neuronal cells, TRPCs contribute to regulating neurite outgrowth, axon guidance, and nerve regeneration (Clapham, 2003; Ramsey et al, 2006). For instance, TRPC1 has been shown to control netrin-1 and brain-derived neurotrophic factor (BDNF)-mediated *Xenopus* spinal neuron axon guidance (Wang and Poo, 2005; Shim et al, 2005) and promote neurite extension in postmitotic neurons derived from embryonic stem cells (Weick et al, 2009). TRPC4 has been reported to increase neurite outgrowth in cultured rat dorsal root ganglia neurons and was up-regulated after nerve injury (Wu et al, 2008). TRPC5 has been variably implicated in regulating neurite outgrowth. For example, excessive TRPC5 expression has been shown to retard neurite outgrowth in PC12 and hippocampal neurons (Greka et al, 2003; Hui et al, 2006), whereas TRPC5 potentiated neurite outgrowth in cerebellar granular and hippocampal neurons (Wu et al, 2007; Davare et al, 2009). TRPC3 and TRPC6 are involved in the BDNF-mediated axon turning in rat cerebellar granule cells (Li et al, 2005). In addition, TRPC6 has been reported to facilitate neurite outgrowth in hippocampal neurons (Tai et al, 2008) and the pheochromocytoma 12 (PC12) cell line (Leuner et al, 2007). Importantly, it has been clearly demonstrated that the expression pattern of TRPCs' changes during neuronal development and/or after nerve injury (Zhou et al, 2008; Huang et al,

2007; Tai et al, 2009; Wu et al, 2008). This suggests that alterations in TRPC expression might be important for fine-tuning the rate of neurite outgrowth, a crucial step in establishing neuronal networks. However, the mechanisms controlling TRPC expression levels during neuronal differentiation have not been fully characterized.

PC12 cells, derived from a tumor of rat adrenal chromaffin cells (Greene and Tischler, 1976), are widely used for investigating the mechanisms of nerve growth factor (NGF)-induced neurite outgrowth. In PC12 cells, NGF activates TrkA (Hartman et al, 1992; Smeyne et al, 1994), a receptor tyrosine kinase, and p75^{NTR}, a neurotrophin receptor which belongs to the tumor necrosis factor receptor subfamily (Reichardt, 2006). TrkA stimulation leads to the activation of PLC γ and the phosphorylation of Erk1/2 (Nusser et al, 2002), whereas p75^{NTR} activation induces NF- κ B signaling (Wood, 1995; Carter et al, 1996; MacDonald et al, 1999; Reichardt, 2006). Importantly, we and others have demonstrated that PC12 cells express several TRPC channels, such as TRPC1, TRPC5, and TRPC6 (Tesfai et al, 2001; Hu et al, 2009). Moreover, the expression pattern of TRPC changes during NGF-induced differentiation in PC12 cells. For instance, Tesfai et al. (Tesfai et al, 2001) demonstrated that the TRPC6 expression level was up-regulated during NGF-induced differentiation of PC12 cells. Therefore, PC12 cells represent an excellent model system for investigating the role of TRPCs during NGF-induced neurite outgrowth.

In this study, we focused on investigating the mechanisms regulating the expression of TRPCs in NGF-differentiated PC12 cells and establishing the role of distinct TRPC subunits during neurite outgrowth in the cells. We confirmed that TRPC6 expression was up-regulated in NGF-differentiated PC12 cells as described earlier (Tesfai et al, 2001). In addition, we found for the first time that NGF pretreatment markedly up-regulated TRPC1 expression and down-regulated TRPC5 expression in PC12 cells. We also demonstrated that NGF increased TRPC1 and TRPC6 expression via an IKK₂-dependent pathway that did not involve TrkA in PC12 cells. Unexpectedly, we found that TRPC1 and TRPC6 affected neurite outgrowth in an opposite manner, with TRPC1 facilitating and TRPC6 slowing down neurite extension. NGF also up-regulated TRPC1 and TRPC6 expression via an IKK₂ pathway in hippocampal neurons, indicating that this pathway may be important for regulating TRPC expression not only in PC12 cells but also in other neuronal systems.

MATERIALS AND METHODS

PC12 cell culture and electroporations

PC12 cells were purchased from ATCC (Cat# CRL-1721). The cells were maintained in the F12K medium (ATCC) supplemented with 15% horse serum (GIBCO) and 2.5% fetal bovine serum (GIBCO). No antibiotics were added to the complete medium. The cells were passaged using a subcultivation ratio of 1:2 twice weekly. Versene (GIBCO) was used to dislodge PC12 cells from the flask surface. The cell suspension was plated on glass coverslips or Petri dishes (BD Biosciences, Bedford, MA) coated with Matrigel (BD Biosciences, Bedford, MA) in Eagle's MEM (ATCC) supplemented with 1% BSA (MEM-BSA medium). The cells were cultured in a humidified 5% CO₂ – 95 % air atmosphere at 37°C. NGF (100 ng/ml, Invitrogen, CA) was used to differentiate PC12 cells. DNA constructs were electroporated into PC12 cells using the Eppendorf multiporator. 4 mm gap

width/800 μ l volume electroporation cuvettes (Eppendorf, Hamburg, Germany) were utilized during electroporations. Exponential decay 850 V pulses of 100 μ s length were used to reversibly electroporabilize PC12 cells. The constitutively active mutant of IKK₂ was obtained from Addgene (plasmid #11105, made by Dr. Anjana Rao (Mercurio et al, 1997)).

Primary culture of rat hippocampal neurons

All animal experimental procedures were performed in accordance with the ethical principles and guidelines of the NIH. The animal protocol was approved by the Institutional Animal Care and Use Committee at Indiana University School of Medicine. Primary cultures of hippocampal neurons were prepared from postnatal day 1 rat pups according to previously described procedures (Dubinsky, 1993). Neurons were plated on glass coverslips coated with Matrigel (BD Biosciences, Bedford, MA) without preplated glia. Neuronal cultures were maintained in a humidified 5% CO₂ – 95% air atmosphere at 37°C in Earl's MEM supplemented with 1% BSA. Cultured hippocampal neurons grown for 24–48 hours *in vitro* were used for all experiments.

Bright field and Fluorescence imaging

A monochromator-based Till-Photonics imaging system (TILL-Photonics, Martinsreid, Germany) equipped with a DU885 Andor CCD camera was used for bright field imaging and monitoring the fluorescence of GFP. The GFP fluorescence was excited at 488 nm. Emitted light was collected with a 510-nm long pass filter. Data were analyzed using TILLvisION software. The determination of the length and number of neurites per cell was performed through the analysis of microscopic images using the Adobe Photoshop CS5 ruler tool. The total length of all neurites in each PC12 cell was determined in 5 to 10 images per experiment and averaged to yield the neurite length per cell value.

RT-PCR

Total RNA was isolated from the control and NGF-pretreated (48–72 hours) PC12 cells by using the SV total RNA isolation kit (Promega). To prevent genomic DNA contamination, the isolated total RNA samples were treated with DNase I. In addition, to further ensure that genomic DNA did not serve as a template, we used primer sets amplifying longer transcripts that span several introns. Each RT-PCR reaction contained 50 ng of the total RNA. One-step RT-PCR Superscript III with Platinum Taq High Fidelity kit (Invitrogen, Carlsbad, CA) was used to amplify TRPC transcripts (30 cycles to amplify TRPC1 and β -actin, and 40 cycles to amplify TRPC5 and TRPC6, (Hu et al, 2009)). The annealing temperature was 57°C. RT-PCR products were resolved on a 0.8% agarose gel stained with ethidium bromide. Relative quantification of TRPC channel expression was done by normalizing the fluorescence intensities of TRPC band to those of β -actin. A 1.5 times larger volume of TRPC5 and TRPC6 PCR products than that of TRPC1 product was loaded into the agarose gel. The RT-PCR data were validated using quantitative real time PCR as described in Hu et al. (Hu et al, 2009). The identity of transcripts was confirmed by sequencing. Several control polymerase chain reactions were performed without an RT step. These experiments resulted in no transcript amplification.

Western blots

Protein lysates were centrifuged at 20,000g for 30 min at 4°C and equal amounts of the proteins (120 µg) were loaded into each well of a 10% SDS-acrylamide/Bis-acrylamide gel (Tris-Glycine running buffer). The resolved proteins were transferred onto nitrocellulose membranes using a Bio-Rad wet transfer apparatus (overnight at room temperature). Nitrocellulose membranes were blocked with 5% filtered non-fat milk and then incubated with either monoclonal 1F1 mouse TRPC1 antibody (1:2800 dilution; a generous gift from Leonidas Tsiokas (Ma et al, 2003)), polyclonal rabbit TRPC5 antibody (1:1000; Alomone Labs), or polyclonal rabbit TRPC6 antibody (1:750; Alomone Labs) overnight at 4°C. After incubation, the membranes were washed and further incubated with an anti-mouse or anti-rabbit secondary antibodies conjugated with the horseradish peroxidase (Pierce, Rockford, IL; 1:20,000) for one hour. The membranes were developed by using a SuperSignal West Femto Kit (Pierce, Rockford, IL) according to the manufacturer's instructions.

Patch-clamp electrophysiology

All electrophysiological experiments were performed using the whole-cell patch-clamp mode as described elsewhere (Obukhov and Nowycky, 2005; Obukhov and Nowycky, 2008). Briefly, currents were recorded during 300 ms ramps from -100 mV to +100 mV applied from a holding potential of -60 mV using the Optopatch amplifier controlled by PCLAMP 10 software (Molecular Devices, CA). Currents were sampled at 1 kHz and filtered at 3 kHz. Series resistance compensation was set to 70%. Currents were activated by the dialysis of GTPγS via the recording pipette (500 µM, Calbiochem, San Diego, CA). The pipette solution contained (in mM): 140 CsMeSO₃; 10 EGTA; 2 MgCl₂; 3.77 CaCl₂; 10 HEPES. The standard extracellular solution contained (in mM): 145 NaCl; 2 CaCl₂; 1 MgCl₂; 2.5 KCl; 10 HEPES; 5.5 Glucose. pH of all solutions were adjusted to 7.2. The PCLAMP 10 software package (Molecular Devices, Sunnyvale, CA) was used for data analysis. Only recordings with series resistance of less than 10 MΩ were analyzed.

Statistics

Data were compared using the t-test followed by the Mann-Whitney Rank Sum test, or one-way ANOVA followed by Dunn's *post hoc* test. The significance level was set to 0.05. Data were expressed as mean ± SEM.

RESULTS

NGF pretreatment alters the pattern of TRPC expression in PC12 cells

PC12 cells were round or slightly elongated when maintained in the MEM-BSA serum-free medium but formed long neurites within 48–72 hours when the medium was supplemented with 100 ng/ml NGF (NGF-differentiated PC12 cells, Figure 1A). To determine how NGF affects the expression of TRPCs we isolated total RNA from both control, undifferentiated, and NGF-treated, differentiated (48–72 hrs) PC12 cells and performed RT-PCR analysis. Similar to adrenal chromaffin cells (Hu et al, 2009; Matsuoka et al, 2009), undifferentiated PC12 cells predominantly expressed TRPC1 and TRPC5 transcripts, but much smaller amounts of TRPC6 transcripts (Figure 1B, C). In control, undifferentiated PC12 cells, the

expression level of TRPC1 RNA was the highest: only 30 PCR cycles were required to amplify the transcript whereas at least 40 cycles were needed to amplify TRPC5 and TRPC6. The NGF-differentiated PC12 cells had significantly higher expression levels of TRPC1 and TRPC6 transcripts (Figure 1B, C). In contrast, the expression level of TRPC5 transcripts was significantly reduced in NGF-differentiated PC12 cells.

We also determined whether changes in the TRPC RNA transcript amounts were accompanied by similar changes in the TRPC protein content in control versus NGF-differentiated PC12 cells. Western blot analysis was used to assess TRPC protein expression levels. The specificity of TRPC antibodies was confirmed using recombinant TRPC proteins expressed in HEK cells (Supplemental Figure 1A–C). Figures 2A–D show a significant increase in TRPC1 and TRPC6 protein expression and a decrease in TRPC5 protein expression in NGF-treated PC12 cells compared to control cells.

TRPC1 channels promote whereas TRPC5 and TRPC6 channels attenuate NGF stimulated neurite outgrowth

To investigate the role of TRPC channels in modulating neurite outgrowth, we electroporated PC12 cells with mixtures of YFP and TRPC expression plasmids. The electroporated cells were cultured in the absence or presence of NGF for 48hrs, and then the total neurite length per cell was determined as described in the Methods. Overexpression of YFP and pcDNA3 (YFP+pcDNA3), YFP and TRPC1 (YFP+TRPC1), YFP and TRPC5 (YFP+TRPC5), or YFP and TRPC6 (YFP+TRPC6) in PC12 cells did not stimulate neurite outgrowth in the absence of NGF (YFP in Figure 3A and data not shown). However, abundant neurite outgrowth was observed in YFP+pcDNA3 expressing PC12 cells in the presence of NGF (control, YFP+NGF, Figure 3A). YFP+TRPC1 overexpression significantly increased the total neurite length per cell (TRPC1+NGF, Figure 3A) as compared to YFP+pcDNA3 expressing PC12 cells, suggesting that elevated TRPC1 expression promoted neurite outgrowth in NGF-differentiated PC12 cells. In contrast, the neurites were significantly shorter in YFP+TRPC5 overexpressing PC12 cells treated with NGF (TRPC5+NGF, Figure 3A), suggesting that excessive expression of TRPC5 negatively regulates neurite outgrowth. Conversely, neurite outgrowth was markedly facilitated in NGF-differentiated, TRPC5-deficient PC12 cells overexpressing an shRNA-TRPC5-GFP construct (OriGene, Rockville, MD; Supplemental Figure 2A–2C) and reduced in NGF-differentiated, TRPC1-deficient PC12 cells overexpressing shRNA-TRPC1-GFP (OriGene, Rockville, MD; Supplemental Figure 2D).

While assessing the effect of TRPC5 overexpression on neurite outgrowth in NGF-differentiated PC12 cells, we observed that the majority of YFP+TRPC5 electroporated cells exhibited no or shorter neurites. However, we also noted that about 30% of YFP+TRPC5 electroporated, NGF-differentiated PC12 cells still had very long neurites. Since NGF treatment up-regulated TRPC1 expression in PC12 cells, we reasoned that this might favor the formation of heteromeric channels between endogenous TRPC1 and heterologously overexpressed TRPC5. Heteromeric TRPC1–TRPC5 channels exhibit markedly smaller conductance than homomeric TRPC5 channels (Strubing et al, 2001). Therefore, the formation of heteromers between heterologously expressed TRPC5 and the endogenous

TRPC1 channel might reduce the retarding effect of overexpressed TRPC5 on neurite outgrowth in PC12 cells.

As the TRPC1–TRPC5 heteromeric channel exhibits a distinct current-voltage relationship that is markedly different from the current-voltage relationship of the wild type TRPC5 channel ((Strubing et al, 2001), insets in Figure 4) we used the patch-clamp technique to examine the formation of functional TRPC1–TRPC5 heteromeric channels in YFP-TRPC5 electroporated, NGF-differentiated PC12 cells. We hypothesized that neurite-bearing, YFP +TRPC5 electroporated, and NGF-treated PC12 cells have high expression levels of endogenous TRPC1 that favors heteromeric TRPC1–TRPC5 formation (between endogenous TRPC1 and exogenous TRPC5). To test this hypothesis, we recorded whole-cell currents in NGF-differentiated PC12 cells overexpressing TRPC5 during intra-patch pipette dialysis with GTP γ S, which can activate TRPC5 and/or TRPC1–TRPC5 channel activity in a receptor independent manner (Obukhov and Nowycky, 2004;Obukhov and Nowycky, 2008). Indeed, we found that the round YFP+TRPC5-expressing cells that lacked neurite outgrowth, exhibited cation currents with the biophysical properties resembling those of homomeric TRPC5 channels. In contrast, the neurite-bearing YFP+TRPC5-expressing PC12 cells exhibited small outwardly-rectifying cation currents that resembled those of heteromeric TRPC1–TRPC5 channels (Figure 4).

We next investigated whether TRPC6 modulates neurite outgrowth in NGF-differentiated PC12 cells. YFP+TRPC6 overexpression resulted in a reduction of the length of neurites, although, to a lesser degree than was observed with YFP+TRPC5 overexpression (TRPC6+NGF, Figure 3A). To further investigate the role of TRPC6 during NGF-induced neurite outgrowth, we mutated the TRPC6 pore helix residues LFW to AAA to generate a dominant negative TRPC6 construct (as described in (Hofmann et al, 2002)), and examined whether the overexpression of this construct affects neurite length in NGF-differentiated PC12 cells. We found that overexpression of YFP+DN-TRPC6 resulted in significantly longer neurites than those observed in control PC12 cells treated with NGF for 48h (DN-TRPC6+NGF, Figure 3A).

We also tested whether GFP-tagged TRPC6 (GFP-TRPC6) and/or DN-TRPC6 (GFP-DN-TRPC6) channel overexpression modulates the NGF-induced neurite formation in PC12 cells. GFP-TRPC6 and GFP-DN-TRPC6 overexpression did not lead to neurite formation in control undifferentiated PC12 cells. In agreement with the results described above, the overexpression of GFP-TRPC6 resulted in a reduction of NGF-induced neurite formation, whereas GFP-DN-TRPC6 overexpression facilitated neurite extension in NGF treated PC12 cells as compared to control GFP electroporated NGF treated PC12 cells (Figure 3B, C). Thus, excessive expression of TRPC6 negatively regulated the length of neurites in NGF-treated PC12 cells, whereas down-regulation of the functional activity of endogenous TRPC6 by heteromerization with DN-TRPC6 promoted neurite outgrowth.

Hyperforin decreases the expression of TRPC6 channels in NGF-differentiated PC12 cells

As hyperforin is known to promote neurite outgrowth, we postulated that it may do so in part by modulating the expression levels of TRPC channels in PC12 cells. We confirmed that 10 μ M hyperforin (Sigma) was effective in potentiating NGF-induced neurite outgrowth

in PC12 cells (Figure 5A). In contrast, a lower concentration of hyperforin (1 μ M) was ineffective. Hyperforin (1–10 μ M) did not induce neurite outgrowth in undifferentiated PC12 cells (Figure 5A). Results of RT-PCR analysis revealed that 1 μ M hyperforin significantly decreased TRPC6 expression in NGF-differentiated PC12 cells without altering expression of either TRPC1 or TRPC5 channels (Figures 5B, C, E). At higher concentrations, hyperforin not only markedly downregulated TRPC6 expression (Figure 5D, E), but also significantly reduced TRPC1 expression and prevented NGF-induced down-regulation of TRPC5 expression. (Figure 5B).

Which of the NGF-induced signaling pathways is important for TRPC up-regulation?

In PC12 cells, NGF stimulates both TrkA and p75^{NTR} receptors. TrkA stimulation results in intracellular Ca^{2+} increases due to the release of Ca^{2+} from intracellular stores and Ca^{2+} influx with a consequent activation of the Calcineurin-NFAT pathway or the Erk1/2-cMyc/Elk1 pathway. On the other hand, p75^{NTR} stimulation results in the activation of the IKK₂-NF- κ B transcription factor signaling cascade (Wood, 1995;Carter et al, 1996;MacDonald et al, 1999;Reichardt, 2006). To determine if TrkA mediates the up-regulation of TRPC1 and TRPC6, we preincubated PC12 cells with NGF in the absence or presence of GW441756 (500 nM), a potent TrkA antagonist, and analyzed neurite formation and TRPC expression. GW441756 did not prevent the NGF-induced up-regulation of TRPC1 and TRPC6 expression (Figure 6A and 6B, left), suggesting that TrkA receptor activity is not responsible for up-regulating TRPC1/TRPC6 expression. GW441756 also failed to significantly affect the total neurite length per cell (Figure 6B, right), suggesting that TrkA is also not required to mediate NGF stimulated neurite outgrowth.

We used two different IKK₂ inhibitors, BMS-345541 and IMD-0354, to examine the contribution of the p75^{NTR}-IKK₂ pathway in regulating TRPC expression in NGF-differentiated PC12 cells. We found that NGF-induced neurite outgrowth was significantly suppressed in the presence of either of these inhibitors (Figure 7A). In addition, these IKK₂ inhibitors prevented TRPC1 and TRPC6 up-regulation in NGF-treated PC12 cells (Figure 7B, 7C and Supplemental Figure 1D). The inhibitor pretreatment did not significantly alter the expression of TRPC channels in control, undifferentiated cells (Figure 7C).

To further confirm the role of the IKK₂ pathway in regulating TRPC expression in PC12 cells, we tested whether an overexpressed, constitutively active mutant of IKK₂ (S177E/S181E, IKK_{2ca}) would be capable of up-regulating TRPC1 and TRPC6 in control PC12 cells in the absence of NGF. We electroporated PC12 cells with the IKK_{2ca} construct and found that in agreement with a previous report (Azoitei et al, 2005), cells over-expressing IKK_{2ca} exhibited neurite outgrowth in the absence of NGF (Figure 8A, B). In addition, the IKK_{2ca}-overexpressing PC12 cells had a significantly elevated expression of TRPC1 and TRPC6 channels in the absence of NGF (Figures 8C–G). In IKK_{2ca}-overexpressing PC12 cells, the TRPC5 expression level was not altered as compared to control PC12 cells transfected with the YFP plasmid (Figure 8F). These data suggest that NGF up-regulates TRPC1 and TRPC6 in NGF-differentiated cells via an IKK₂-dependent pathway.

Does NGF regulate neurite outgrowth and TRPC channel expression through a p75^{NTR}-IKK2 pathway in hippocampal neurons?

We next investigated whether NGF modulates neurite outgrowth and TRPC expression in the primary culture of hippocampal neurons. In contrast to PC12 cells, hippocampal neurons spontaneously formed neurites when cultured longer than 24 hours, especially in dense cultures, probably due to the release of endogenous growth factors. Therefore, we first examined the effect of NGF on neurite length and TRPC expression in sparsely plated hippocampal neurons that were cultured for 24–30 hours. Figure 9 shows that the neurite length was increased in NGF pretreated hippocampal neurons. To assess whether NGF modulated the expression of TRPC channels in hippocampal neurons, we performed RT-PCR analysis as described above (Figure 9C). We found that hippocampal neurons predominantly expressed TRPC1, TRPC5, and TRPC6 channels. Interestingly, like in PC12 cells, the expression level of TRPC1 and TRPC6 was significantly increased, whereas TRPC5 expression was significantly decreased in NGF-treated hippocampal neurons (Figure 9D). The NGF-dependent increases in TRPC expression were abolished in the presence of IMD-0354 (0.5 μ M), confirming the involvement of the IKK₂-dependent pathway in hippocampal neurons.

We also tested whether DN-TRPC6 would promote NGF-stimulated neurite outgrowth in cultured hippocampal neurons. We electroporated freshly isolated hippocampal neurons with mixtures of YFP+pcDNA3 (control) or YFP+DN-TRPC6 plasmids, and determined the length of neurites 48 hrs following electroporation. These experiments showed that DN-TRPC6 over-expression significantly facilitated neurite extension in primary cultured hippocampal neurons (Figure 10).

DISCUSSION

The main findings of our study are that: (1) NGF up-regulated not only TRPC6, but also TRPC1 expression and down-regulated TRPC5 expression; (2) TRPC1 decreases the retarding effect of TRPC5 on neurite outgrowth in part by forming TRPC1–TRPC5 heteromeric channels; (3) NGF up-regulated TRPC1 and TRPC6 expression via a p75^{NTR}-IKK₂-dependent pathway that did not involve TrkA receptor; (4) despite the fact that NGF up-regulated both TRPC1 and TRPC6 expression, only TRPC1 potentiated neurite outgrowth whereas TRPC6 decelerated neurite extension.

TRPC channels and neurite outgrowth

Although it is well established that TRPC channels are involved in regulating neurite outgrowth, the specific functions of different TRPC channel isoforms is controversial. One of the limitations of these previous studies is that the functions of individual TRPC isoforms were examined in numerous different cell systems. For instance, Greka et al. and Hui et al. (Greka et al, 2003; Hui et al, 2006) demonstrated that excessive TRPC5 expression retarded neurite outgrowth in PC12 and hippocampal neurons. In contrast, Wu et al (Wu et al, 2007) and Davare et al. (Davare et al, 2009) reported that TRPC5 was essential for initiating neurite outgrowth in cerebellar granular and hippocampal neurons, respectively. To help resolve these differences we analyzed the expression of all TRPC channels found in PC12

cells and then determined the functions of each of the isoforms that were expressed at significant levels (TRPC1, 5, 6). In support of the conclusions of Greka et al. and Hui et al. (Greka et al, 2003;Hui et al, 2006), we also observed that TRPC5 plays a negative role in regulating neurite outgrowth in PC12 cells (Figure 3A and Supplemental Figure 2A–C). Consistent with the results of Weick et al. (Weick et al, 2009), who demonstrated that TRPC1 promoted neurite extension in postmitotic neurons derived from embryonic stem cells, we also found that TRPC1 promotes neurite outgrowth in NGF-differentiated PC12 cells. Interestingly, our electrophysiological data indicate that endogenous TRPC1 may prevent the inhibitory effect of overexpressed TRPC5 on NGF-induced neurite outgrowth in PC12 cells, by forming heteromeric TRPC1–TRPC5 channels (Figure 4). We propose that NGF-induced increases in TRPC1 may drive heteromeric TRPC1–TRPC5 channel formation that acts together with the decreases in TRPC5 expression to facilitate neurite outgrowth during the initial stages of NGF-induced differentiation. The heteromeric TRPC1–TRPC5 channel exhibits markedly reduced permeability for cations as compared to the homomeric TRPC5 channel (Strubing et al, 2001). Thus, TRPC1 and TRPC5 heteromerization may limit TRPC5-mediated cation and Ca^{2+} influx in NGF-differentiated PC12 cells. We speculate that optimal neurite outgrowth might require very specific amounts of Ca^{2+} entry, with excessive Ca^{2+} influx retarding and moderate Ca^{2+} influx facilitating neurite outgrowth.

This hypothesis is also consistent with our data showing that TRPC6, a Ca^{2+} -permeable channel, decreases neurite outgrowth in PC12 cells and hippocampal neurons (Figure 2 and Figure 10). However, these data are not consistent with previous studies that suggested that TRPC6 facilitates neurite outgrowth in hippocampal neurons (Tai et al, 2008) and PC12 cells (Leuner et al, 2007). In part this conclusion was based on the observation that hyperforin, an activator of TRPC6 was shown to potentiate neurite outgrowth in a subclone of PC12 cells (Leuner et al, 2007;Leuner et al, 2010). Indeed we also observed that hyperforin increases neurite outgrowth (Figure 5A), although while doing so it decreased TRPC6 expression in NGF-differentiated PC12 cells (Figure 5B–E). Although our data do not rule out the possibility that hyperforin may still be activating the remaining TRPC6 channels, it would suggest that a reduction of TRPC6 expression rather than hyperforin-induced TRPC6 activation may be responsible for promoting neurite outgrowth in PC12 cells. This proposal is supported by data showing that overexpression of TRPC6 attenuates neurite outgrowth whereas overexpression of a dominant negative TRPC6 promotes outgrowth (Figure 3). The mechanism by which hyperforin down-regulates TRPC6 expression in PC12 cells is unclear at this time. It is unlikely that TRPC6 stimulation is responsible for its down-regulation. It is more likely that off-target effects of hyperforin such as the release of mitochondrial Ca^{2+} and Zn^{2+} account for TRPC6 down-regulation (Tu et al, 2010;Gibon et al, 2010).

The negative effect of TRPC6 on neurite outgrowth described in our study does not exclude a possible involvement of TRPC6 in either the guidance of neurites regulated by BDNF (Li et al, 2005) or in spine formation (Zhou et al, 2008). We did not observe spine formation in NGF-differentiated PC12 cells and hippocampal neurons usually exhibit spine formation between 4 and 28 days after birth (Zhou et al, 2004;Nimchinsky et al, 2002), whereas we studied only postnatal day one hippocampal neurons in short-term (2 days) primary cultures.

Interestingly, in the developing rat brain, spine formation and synaptogenesis overlap between postnatal day 7 and 28 (Zhou et al, 2008; Nimchinsky et al, 2002), a time when TRPC6 expression levels also peak (Zhou et al, 2008). Importantly, TRPC6 protein was found in synaptosomes and postsynaptic membranes of dendritic excitatory synapses, suggesting a role of TRPC6 during synaptogenesis (Zhou et al, 2008). This function agrees well with our conclusion that TRPC6 may be important for decelerating neurite outgrowth because neurite extension should be slowed to ensure proper synapse formation. We propose that interplay between the acceleration (TRPC1-mediated) and deceleration (TRPC6-mediated) of dendrite/axon elongation may be important for establishing neuronal networks.

The role of the NF- κ B cascade in regulating TRPC expression

Given the importance of TRPCs in neuritogenesis, it was pivotal to identify the signaling pathway governing the channels' expression in differentiating neuronal cells. Such information is valuable in a drug discovery process aimed at designing new therapeutic tools to treat neurodegenerative diseases. In the current study, we demonstrated that NGF activation of IKK₂ is required to mediate the effects of NGF on TRPC1 and TRPC6 expression in PC12 cells and hippocampal neurons. IKK₂ is a kinase that phosphorylates the inhibitor of κ B (I κ B) releasing it from NF- κ B, and thereby allowing NF- κ B to translocate into the nucleus. It has been previously shown that NGF-stimulates translocation of NF- κ B into the nucleus of PC12 cells (Wood, 1995). Together these data suggest that the NF- κ B cascade is involved in up-regulating TRPC1 and TRPC6 expression in PC12 cells. In support of this, it has been reported that tumor necrosis factor α up-regulates TRPC1 expression in endothelial cells via an NF- κ B-dependent pathway (Paria et al, 2003). However, we cannot rule out the possibility that IKK₂ regulates TRPC expression through an NF- κ B independent mechanism (Chariot, 2009). Interestingly, a similar IKK₂-dependent mechanism was important for up-regulating TRPC1 and TRPC6 expression in rat hippocampal neurons. Although NGF increased the rate of neurite elongation in hippocampal neurons, as these neurons form neurites in the absence of NGF it is obvious that other factors may also significantly contribute to modulating neuritogenesis in this model.

NGF-induced TRPC5 down-regulation still occurred in the presence of both tested IKK inhibitors (BMS-345541 and IMD-0354) or after IKK_{2ca} overexpression. These findings suggest that an IKK₂-independent signaling pathway must be responsible for NGF-mediated down-regulation of TRPC5. Interestingly, the TRPC6 activator hyperforin prevented the NGF-induced down regulation of TRPC5 (Figure 5). Further experiments are needed to unravel the mechanisms underlying NGF-induced TRPC5 down-regulation.

Do the observed phenomena play a role in adrenal gland physiology?

PC12 is a tumor cell line derived from rat adrenal chromaffin cells, a neuroendocrine cell type. Phenotypically PC12 cells resemble fetal chromaffin cells (Duman et al, 2008), which originate in the neural crest. During embryonic development fetal chromaffin cells migrate along nerve fibers to the adrenal cortex to form the adrenal medulla. It has been suggested that neurite formation involves similar mechanisms to those that drive cell migration (Mattila and Lappalainen, 2008). Therefore, it is possible that in a fetus, NGF or an NGF-

like molecule released from nerve fibers serves as a guidance cue for migrating chromaffin cells on their way to the adrenal cortex. We speculate that TRPC1, TRPC5, and TRPC6 channels might play a role in regulating the rate and direction of migration of fetal chromaffin cells.

Conclusions

In summary, we have demonstrated that NGF up-regulated TRPC1 and TRPC6, but down-regulated TRPC5 expression. We showed that NGF up-regulates expression of TRPC1 and TRPC6 via an IKK₂ dependent pathway in PC12 cells and hippocampal neurons (Figure 11). Surprisingly, elevated TRPC1 and TRPC6 expression modulated neurite outgrowth in opposite ways. Increased TRPC1 promoted neurite extension, whereas elevated TRPC6 decreased neurite outgrowth. These data suggest that the rate of neurite outgrowth is determined by a balance between TRPC1, TRPC5, and TRPC6 expression and alterations in the expression balance is accountable for the observed changes in the rate of neurite outgrowth. We propose that TRPC1 is a driving force for accelerating neurite outgrowth, whereas TRPC6 serves as an NGF-dependent “molecular damper” maintaining a submaximal velocity of neurite extension. We also propose that TRPC1 overexpression and/or the genetic or pharmacological inactivation of the TRPC6 channel may help combat neurodegenerative diseases and act as a useful therapy for facilitating neurite outgrowth and/or axon regeneration in neuronal pathologies associated with nerve injuries.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

TRPC	Transient Receptor Potential Canonical
NGF	Nerve growth factor
BDNF	Brain-derived neurotrophic factor
PC12	Pheochromocytoma 12 cell line
TrkA	Neurotrophic tyrosine kinase receptor type 1
p75^{NTR}	p75 Neurotrophin receptor
IKK₂	Inhibitor of κ B kinase
Erk1/2	Extracellular Signal-Regulated Kinases 1 and 2

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Figure 1.

Comparison of TRPC expression levels in control and NGF-differentiated PC12 cells. **A.** Representative bright field images of undifferentiated (control) and NGF-differentiated (NGF) PC12 cells are shown. **B.** RT-PCR analysis of TRPC expression in PC12 cells. PCR products amplified using TRPC specific primer sets were separated on ethidium bromide stained agarose gels. A representative gel image is shown. **C.** Comparison of TRPC expression in control (C) and NGF-differentiated PC12 cells (NGF). Each experiment was repeated 7–9 times.

Figure 2.

Western blot analysis of TRPC protein expression in PC12 cells. **A.** Monoclonal anti-TRPC1 antibody (a generous gift from Dr. Leonidas Tsiokas) was used to detect the TRPC1 protein in control (C) and NGF-differentiated PC12 cells (NGF). The specificity of monoclonal anti-TRPC1 was confirmed using mouse recombinant TRPC proteins. **B.** TRPC5 protein expression was detected only in undifferentiated PC12 cells. **C.** NGF pretreatment up-regulated TRPC6 in PC12 cells. In **A–C**, the lanes labeled mTRPC1, mTRPC5, and hTRPC6 represent recombinant proteins used as controls. “C” denotes

control, protein extracts of untreated PC12 cells. “NGF” labels protein extracts isolated from PC12 cells treated with NGF for 48 hrs. **D.** Comparison of relative TRPC protein expression levels in control and NGF-differentiated PC12 cells. Each experiment was repeated 10–16 times.

Figure 3.

DN-TRPC6 facilitates NGF-induced neurite outgrowth in PC12 cells. **A.** Comparison of total neurite length per cell in PC12 cells electroporated with a mixture of the YFP plasmid and the pcDNA3 plasmid with or without an insert as indicated. Each group consisted of 35 to 312 cells. **B.** Comparison of total neurite length per cell in PC12 cells electroporated with GFP-tagged TRPC construct as indicated. **C.** Representative images demonstrating the effect of GFP-TRPC6 and DN-GFP-TRPC6 overexpression on the total neurite length per cell. In these pseudocolor images, the red color corresponds to the highest YFP fluorescence, whereas the blue color indicates the lowest YFP fluorescence.

Figure 4.

Electrophysiological characterization of GTP γ S-activated currents in TRPC5-overexpressing cells. **A.** Currents with TRPC5-like current-voltage relationships were observed in the round TRPC5 expressing PC12 cells (n=8). **B.** Heteromeric TRPC1–TRPC5 current-voltage relationships were seen in the TRPC5-overexpressing PC12 cells exhibiting neurites (n=8). The insets in the quadrant II show representative images of TRPC5-expressing PC12 cells, whereas the insets in the quadrant IV show sample current-voltage relationships of the homomeric TRPC5 channel or the TRPC1–TRPC5 heteromeric channel

heterologously expressed in HEK cells. The solid line corresponds to the averaged current amplitudes, whereas the vertical bars represent the SEM.

Figure 5.

The effect of hyperforin on neurite outgrowth and TRPC expression in NGF-differentiated PC12 cells. **A**. Comparison of the total neurite length per cell in hyperforin-treated PC12 cells (n=405–635). **B**, **C**, and **D**. Hyperforin-induced changes in relative TRPC channel expression in PC12 cells (n=4 in each group). **E**. Representative images of gels used during the RT-PCR analysis. “HF” = hyperforin. “Control” = undifferentiated PC12 cells. “NGF” = NGF-differentiated PC12 cells.

Figure 6.

Receptor tyrosine kinase TrkA is not involved in the NGF-induced up-regulation of TRPC1 and TRPC6. **A.** RT-PCR analysis of TRPC1 (upper panel) and TRPC6 (lower panel) expression in NGF-treated PC12 cells in the absence or presence of 500 nM GW441756, a potent TrkA inhibitor. **B.** Comparisons of the relative expression levels of TRPC1 and TRPC6 (left, $n = 6-7$), and the total neurite length per cell (right, $n = 276-290$) in NGF treated PC12 cells in the absence or presence of 500 nM GW441756.

Figure 7.

IKK₂ inhibitors abolished NGF-mediated increases of TRPC1 and TRPC6 channel expression in NGF-treated PC12 cells. **A.** Two different IKK₂ inhibitors, BMS (BMS-345541, 10 μM) and IMD (IMD-0354, 1 μM), potentially inhibited neurite outgrowth in NGF-differentiated PC12 cells (n = 94–383). **B.** RT-PCR analysis of TRPC expression in the IKK₂ inhibitor treated NGF-differentiated PC12 cells (upper and middle panel). The lower panel shows the effect of the tested IKK₂ inhibitors on basal TRPC1 and TRPC5 expression levels in control PC12 cells. TRPC6 expression level was very low in control

PC12 cells and was not significantly altered in IKK₂ inhibitor experiments (see summary in C). Representative gels are shown. C. The summary of RT-PCR analysis of relative TRPC expression in IKK₂ inhibitor treated groups. “NGF+BMS” stands for NGF-differentiated PC12 cells treated with (BMS-345541, 10 μM). “NGF+IMD” stands for NGF-differentiated PC12 cells treated with (IMD-0354, 1 μM). “C” denotes undifferentiated PC12 cells. “NGF” denotes NGF differentiated PC12 cells. Each experiment was repeated 4–13 times.

Figure 8.

The effect of overexpression of a constitutively active mutant of IKK₂ (S177E/S181E, IKK_{2ca}) on TRPC channel expression in PC12 cells. **A.** IKK_{2ca}-overexpressing undifferentiated PC12 cells exhibited longer neurites than non-transfected cells at 48 hrs post-electroporation time. Shown is an overlay of the fluorescence and corresponding bright field images. The YFP fluorescence is shown in pseudo-color. **B.** Comparison of the total neurite length per cell in pcDNA3 and IKK_{2ca}-overexpressing undifferentiated PC12 cells ($n = 231-267$). **C** and **D** show the RT-PCR analysis of TRPC1 and TRPC6 expression in PC12

cells expressing either pcDNA3 or IKK_{2ca}. **E, F, and G** show the relative changes in the expression of TRPC channels induced by the overexpression of IKK_{2ca}. Each experiment was repeated 10–12 times.

Figure 9.

Effects of IKK_2 inhibition on the expression of TRPC channels in cultured hippocampal neurons. **A.** Representative images of rat cultured hippocampal neurons maintained either in the control medium, the NGF-containing medium, or the medium supplemented with the mixture of NGF and IMD-0354 (0.5 μM). Similar to PC12 cells, NGF-treated cultured hippocampal neurons exhibited longer neurites (**B**, 24 h incubation, $n = 20-32$). NGF-IMD treated cells showed retarded neurites. **C.** Representative agarose gels used during the RT-PCR analysis of TRPC1 and TRPC6 expression in hippocampal neurons treated with NGF

or NGF+IMD. **D.** Comparison of relative TRPC expression levels in control versus NGF- or NGF+IMD-treated hippocampal neurons. “C” denotes “control.” Each experiment was repeated 3–7 times.

Figure 10.

Effect of DN-TRPC6 overexpression on neurite outgrowth in the primary culture of hippocampal neurons. The electroporated neurons were plated on matrigel-coated coverslips and incubated in MEM-BSA medium supplemented with NGF for 48 hours. **A.** Three pairs of representative images of hippocampal neurons electroporated with the mixtures of either GFP+pcDNA3 (1:23) or GFP+DN-TRPC6 (1:23) cDNAs. GFP fluorescence is indicated in gray inverted scale; non-fluorescing cells are not seen. **B.**

Comparison of total neurite length per cell in GFP+pcDNA3 (n=50) versus GFP+DN-TRPC6 (n=101) electroporated hippocampal neurons.

Figure 11.

The schematic shows two possible signaling pathways that can be triggered by nerve growth factor (NGF) binding. (1) The first pathway includes the Tyrosine Kinase Isoform A (TrkA). TrkA stimulation leads to the activation of Phospholipase C γ (PLC γ) which cleaves Phosphatidylinositol 4,5-bisphosphate (PIP₂) to inositol 1,4,5-triphosphate (IP₃) and diacylglycerol (not shown). IP₃ binds and activates the IP₃-receptor (IP₃-R) on the endoplasmic reticulum (an intracellular calcium store) allowing Ca²⁺ release. Released Ca²⁺ activates Calmodulin (not shown), which in turn stimulates Calcineurin (Caln). Caln dephosphorylates the Nuclear Factor of Activated T-Cells (NFAT) promoting its translocation into the nucleus where NFAT activates gene expression. In addition, TrkA stimulation results in the phosphorylation of Extracellular Signal-Regulated Kinases 1 and 2 (Erk1/2). Phosphorylated Erk1/2 translocate into the nucleus where they phosphorylate the transcription factors: ELK1 and c-Myc. The phosphorylated transcription factors then promote gene expression. In our experiments a TrkA receptor inhibitor GW441756 (GW), did not affect the expression of TRPC1 and TRPC6 channels, suggesting that the channel's expression is independent of this pathway. (2) The second pathway involves the neurotrophin receptor, p75^{NTR}. p75^{NTR} activates the inhibitor of nuclear factor kappa-B

kinase subunit beta (IKK₂). IKK₂ phosphorylates the inhibitor of the nuclear factor kappa-B (I κ B). The phosphorylation of I κ B promotes its dissociation from the NF- κ B dimer. The phosphorylated I κ B is ubiquitinated whereas the nuclear factor kappa-B (NF- κ B) translocates into the nucleus and up-regulates gene expression. We demonstrated that BMS-345541 and IMD-0354, two different IKK₂ inhibitors, prevented the NGF-induced up-regulation of TRPC1 and TRPC6 expression. Contrary, the constitutive active mutant of IKK₂, IKK_{2Ca}, up-regulated the expression of TRPC1 and TRPC6, which supports the hypothesis that TRPC1 and TRPC6 expression is regulated via the p75^{NTR}-IKK₂ pathway but not the TrkA pathway. (3) The up-regulation of TRPC1 positively regulates neurite outgrowth, whereas TRPC6 up-regulation negatively regulates neurite outgrowth which is in agreement with previous reports indicating the role of TRPC6 in synaptogenesis (Zhou et al, 2008).