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Research Report

# Pim-1 kinase enhances NFATc activity and neuroendocrine functions in PC12 cells

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**Abstract**

The activity of NFATc family transcription factors is tightly regulated in T cells via signaling pathways initiated by stimulation of the T cell receptor or its downstream effectors such as the Pim-1 serine/threonine kinase. Here, we demonstrate that NFATc-dependent transcription is inducible also in NGF-differentiated rat PC12 pheochromocytoma cells treated with phorbol esthers, calcium ionophores and/or forskolin and that the Pim-1 kinase can further potentiate the effects of these agents. PC12 cells share many characteristics with sympathetic neurons and can be induced to produce and release catecholamines, such as dopamine and noradrenaline, and inflammatory cytokines, such as interleukin 6. Interestingly, Pim-1 can synergize with forskolin-induced signaling pathways to stimulate also neuroendocrine functions of PC12 cells.
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*Theme:* Neurotransmitters, modulators, transporters and receptors
*Topic:* Second messengers and phosphorylation

*Keywords:* Pim-1; NFATc; NGF; Forskolin; Catecholamine

## 1. Introduction

The nuclear factors of activated T cells consist of four family members (NFATc1–c4) which play crucial roles in regulating expression of immune response genes [6,31]. Yet, NFATc family members are not restricted to the immune system but are also expressed in several other tissues including the brain [4,12,16,28]. In both lymphoid and neuronal cells, transcriptional activity of NFATc proteins can be induced by receptor-mediated protein kinase C (PKC)- and calcium-dependent signaling pathways, which can be mimicked by a combined treatment with phorbol esthers such as PMA and calcium ionophores such as ionomycin. While NFATc proteins enter the nuclei in response to calcium- and calcineurin-dependent dephosphorylation, several protein kinases including cyclic AMP-dependent protein kinase A (PKA) can in turn phosphorylate them and thereby promote their nuclear exit [3,5,29,40]. Accordingly, PKA agonists such as forskolin inhibit NFATc-induced interleukin-2 (IL-2) production in T-cells [34]. We have recently demonstrated that also the Pim-1 kinase can physically interact with NFATc1 and phosphorylate it on several serine residues, but unlike the other known NFATc kinases, Pim-1 enhances NFATc-dependent transactivation and cytokine production [30] without any effects on the subcellular localization of NFATc1 (J. Sandholm, K. Heiskanen and P.J. Koskinen, manuscript in preparation).

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Pim-1 is an oncogenic serine/threonine kinase, whose expression in hematopoietic cells is transiently induced upon activation of cytokine or antigen receptors [7,20,32,39]. We have observed that, during murine embryogenesis, the three *pim* family genes are expressed in overlapping patterns in the central nervous system [8], while in adult brain tissues, the *pim* genes are expressed at lower levels but can be upregulated, e.g. by seizure activity [10,18].

Rat PC12 pheochromocytoma cells are adrenal gland-derived chromaffin-like cells that are widely used as a neuronal cell culture model since they can be differentiated by nerve growth factor (NGF) or interleukin 6 (IL-6) into cells that resemble sympathetic neurons and that are able to synthesize and release catecholamines [14,33]. Catecholamines can in turn induce production of IL-6 in differentiated PC12 cells [9], suggesting that there is an autocrine positive feedback loop, which not only promotes differentiation, but in addition helps the cells to resist apoptotic stimuli [35].

Since Pim and NFATc family proteins are coexpressed in PC12 cells and within the central nervous system and since both types of proteins have been implicated in regulation of synaptic plasticity and memory [12,18], we hypothesized that, similarly to T cells, Pim kinases might enhance NFATc activity also in neurons. Here, we demonstrate that, in NGF-differentiated PC12 cells, Pim-1 potentiates other signaling pathways not only to stimulate NFATc activity, but also to enhance neuroendocrine functions of PC12 cells, as evidenced by increased production of catecholamines.

## 2. Materials and methods

### *2.1. Plasmid constructs*

The pSV-*pim*-1 and pSV-*pimNT81* expression vectors have been described previously [2]. The NFAT-LUC luciferase reporter plasmid kindly provided by G.R. Crabtree (Stanford University, Stanford, CA) contains three copies of the composite NFAT sites derived from the IL-2 enhancer binding both NFATc and AP-1 family members. The NFAT-mut-LUC reporter without functional NFAT sites was derived from NFAT-LUC, as described previously [30], and the pSV-β-galactosidase reporter construct was from Promega (Madison, WI).

### *2.2. Cell culture*

PC12 cells were plated on Primaria six-well plates (BD Biosciences, San Jose, CA) or on collagen-coated plates (Collagen type I from rat tail, Sigma-Aldrich, St. Louis, MO) and grown in DMEM supplemented with 5% fetal calf serum, 10% horse serum and the antibiotics penicillin and streptomycin. The cultures were maintained at 37 °C in a 5% $CO_2$/air atmosphere. Differentiation of PC12 cells was induced by adding NGF (50 ng/ml, Promega) to the culture medium for 4 to 6 days.

### *2.3. Transactivation assays*

NGF-differentiated PC12 cells were transfected by the FUGENE 6 transfection reagent (Roche Diagnostics, Indianapolis, IN) with 1 μg of reporter plasmids together with 1 μg of *pim*-1 expression vectors. Two days after transfection, cells were left unstimulated or were stimulated for 3 to 6 h with 10 ng/ml PMA (Sigma-Aldrich) and 5 μM ionomycin (Calbiochem, La Jolla, CA). To stimulate protein kinase A activity, cells were cotreated with 20 μM forskolin (Sigma-Aldrich), and to inhibit calcineurin activity, 1 μg/ml of cyclosporin A (Sigma-Aldrich) was added. After treatments, cells were collected and analyzed for luciferase activities using the Labsystems luminometer (Labsystems, Helsinki, Finland). The transfection efficiencies were normalized against β-galactosidase activities. Shown in the figures are means and standard deviations of representative experiments with at least triplicate samples. Statistical analyses were carried out using Student's *t* test, where stimulated samples were compared to untreated control samples; * means $P < 0.05$ and $^{**}P < 0.01$.

### *2.4. Western blot analysis*

Cells were lysed into SDS gel loading buffer by heating them at 95 °C for 10 min, and proteins of the lysates were separated by SDS-PAGE followed by transfer into nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). For Western blotting, the membranes were blocked with 3% nonfat dry milk in Tris-buffered saline and incubated with monoclonal anti-Pim-1 (19F7, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-TH (Zymed Laboratories, San Francisco, CA) antibodies or with affinity-purified polyclonal antiserum against NFATc1 (Immunoglobe, Himmelstadt, Germany). The proteins recognized by primary antisera were visualized by using horseradish-peroxidase-conjugated secondary antibodies and ECL+Plus reagents (Amersham Biosciences, Uppsala, Sweden). Equal protein loading was verified by blotting stripped membranes with anti-β-actin antibody (Sigma-Aldrich).

### *2.5. Immunocytochemistry*

Cells were fixed in 4% paraformaldehyde and incubated with primary antisera followed by TRITC-conjugated secondary antibodies (Zymed Laboratories). Specificities of immunoreactivities were confirmed by omitting primary antisera from the reactions.

### *2.6. Catecholamine assays*

Cells were collected in 0.1 M perchloric acid, and the cellular levels of the catecholamines dopamine (DA) and

noradrenaline (NA) and their precursor L-DOPA were determined by electrochemical detection (ESA Coulochem 5011, Bedford, MA) from samples separated by HPLC on a reversed-phase C18 column (Ultrasphere ODS, 4.6 × 250 mm, Beckman Instruments, Fullerton, CA).

## 3. Results

### 3.1. NFATc family members are inducibly regulated in NGF-differentiated PC12 cells

To determine whether NFATc-dependent gene expression can be induced, not only in undifferentiated PC12 cells [4], but also after their differentiation to neuron-like cells, PC12 cells were cultured in the presence of NGF for 4 days and then transfected with a luciferase reporter gene construct containing three NFAT-binding sites derived from the IL-2 enhancer. Two days later, when the cells were stimulated for 3 h with PMA and ionomycin, NFATc activity was stimulated nearly 4-fold above the basal level (Fig. 1A). By contrast, this stimulation was completely lost when cells were cotreated with the calcineurin inhibitor cyclosporin A (CsA).

The relatively high basal NFATc activity observed in PC12 cells suggests that the brain-specific NFATc isoforms are differentially regulated as compared to those expressed in lymphoid cells. When we analyzed by Western blotting the expression levels of the NFATc family proteins in NGF-differentiated PC12 cells, we were able to detect expression of both NFATc2, as shown previously [16,28], and NFATc1 (Fig. 1B and data not shown). Moreover, their expression levels were not significantly changed in cells stimulated for 3 h with PMA, ionomycin and/or forskolin. Immunocytochemical analysis of the cells further confirmed that, upon stimulation, both NFATc1 and NFATc2 proteins had translocated from the cytoplasm to the nucleus (Figs. 1C–D and data not shown). Taken together, our data indicated that NFATc activity can be regulated in the neuron-like PC12 cells in a similar inducible manner as in cells of the immune system.

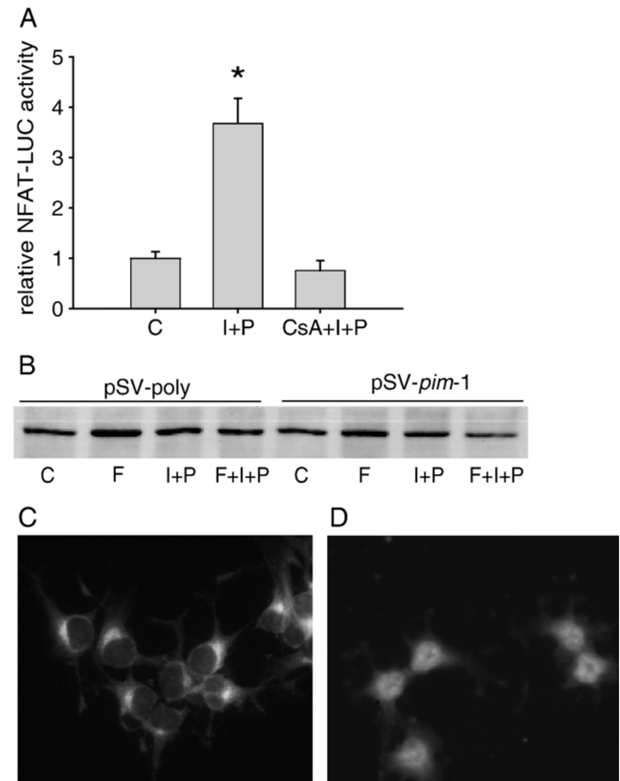


Fig. 1. NFATc family members are inducibly regulated in NGF-differentiated PC12 cells. (A) PC12 cells were differentiated in the presence of NGF and then transiently transfected with NFAT-luciferase (NFAT-LUC) and SV40-driven beta-galactosidase (SV-β-gal) reporter plasmids. Two days later, cells were left unstimulated (C) or stimulated for 3 h with indicated combinations of ionomycin (I), PMA (P) or cyclosporin A (CsA). Cells were collected, lysed and analyzed for reporter enzyme activities. Shown are luciferase activities normalized against beta-galactosidase activities and calculated relative to those obtained from unstimulated control cells. (B) Differentiated PC12 cells were transiently transfected with either pSV-poly or pSV-*pim*-1 expression vectors. Two days later, cells were stimulated for 3 h with indicated combinations of drugs including also forskolin (F), after which NFATc1 proteins levels were analyzed from cell lysates by Western blotting. (C–D) Untransfected PC12 cells were left unstimulated (C) or stimulated with I and P for 3 h (D), after which cells were fixed and subcellular distribution of NFATc1 protein was determined by immunocytochemistry.

### 3.2. Pim-1 enhances NFATc activity in PC12 cells

Since we had previously shown that Pim-1 can stimulate NFATc1-dependent transactivation in lymphoid cells [30] and since PC12 cells express distinct members of the NFATc family as compared to lymphoid cells [16,28], we wanted to analyze the ability of Pim-1 to enhance NFATc activity also in PC12 cells. We transfected NGF-differentiated PC12 cells with an NFAT-luciferase reporter together with an empty vector or vectors expressing either the 33 kDa murine Pim-1 protein or a kinase-deficient NT81 mutant of Pim-1. Two days later, cells were stimulated for 3 h with PMA and/or ionomycin. Similarly to Jurkat T cells [30], also in PC12 cells, wild-type Pim-1, but not the kinase-deficient mutant, further enhanced NFATc activity induced by PMA and ionomycin (Fig. 2A). However, unlike in Jurkat T cells, Pim-1 synergized with the ionomycin-induced pathway also in the absence of PMA, most likely due to the high basal AP-1 activity of PC12 cells. The enhancing effects of Pim-1 could not be explained by any major changes in endogenous NFATc expression since the protein levels of either NFATc1 or NFATc2 were not markedly affected by coexpression of Pim-1 (Fig. 1B and data not shown). When we transfected PC12 cells with reporter constructs containing no or mutated NFAT binding sites, Pim-1 did not significantly affect the low basal luciferase activity observed in either unstimulated or stimulated cells (Fig. 2A and data not shown), suggesting that the effects of Pim-1 were specifically targeted towards NFATc proteins and not mediated by other factors binding to the promoter region of the reporter.

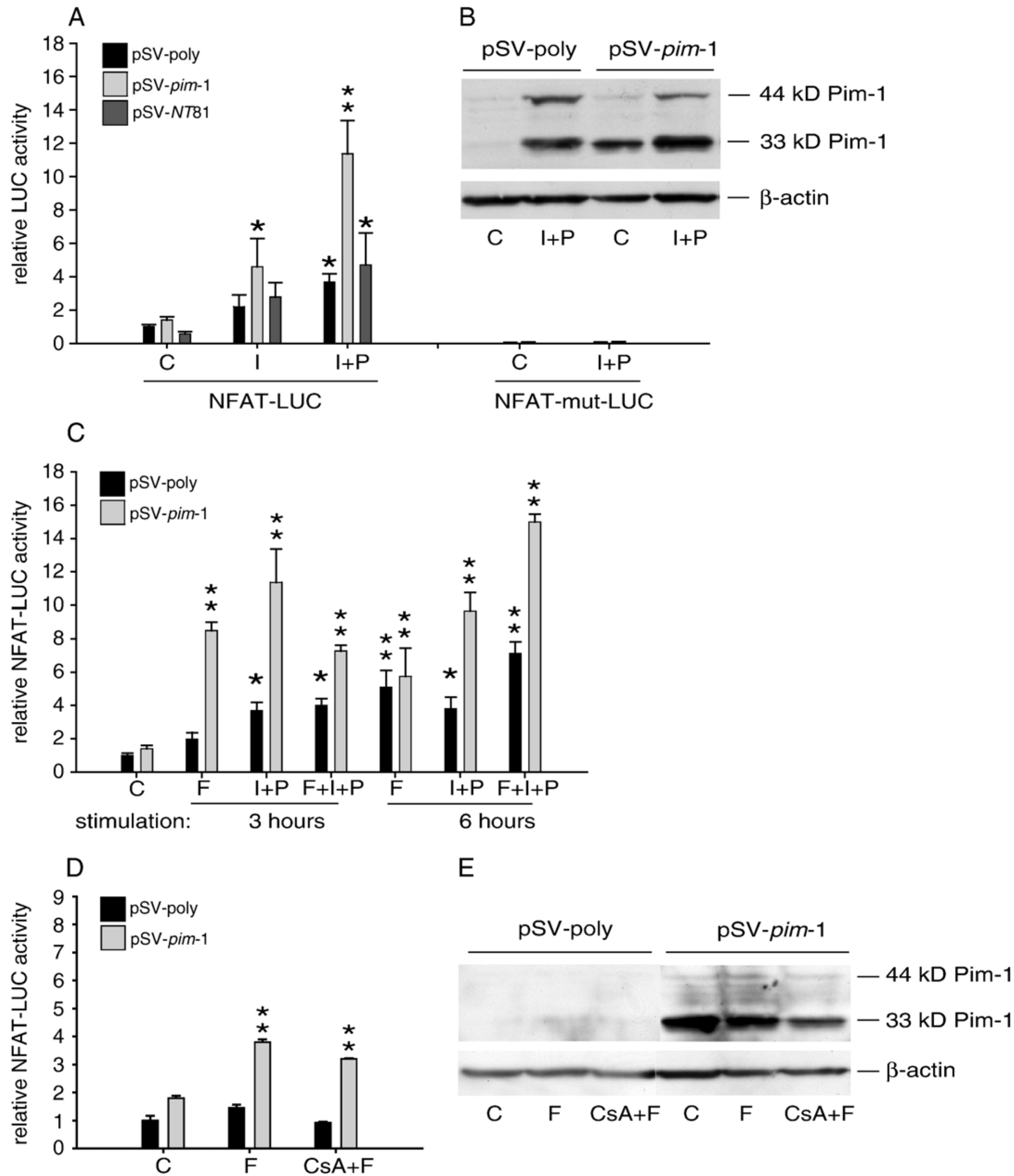


Fig. 2. Pim-1 enhances NFATc activity in PC12 cells. NGF-differentiated PC12 cells were transiently transfected with NFAT-LUC and SV-β-gal reporters together with either pSV-poly, pSV-*pim*-1 or pSV-*pimNT81* expression vectors. Two days later, cells were stimulated for 3 (A–E) or 6 h (C) with indicated combinations of drugs (abbreviated as in Fig. 1), after which relative luciferase activities were determined as above. To control for specificity, a mutated reporter (NFAT-mut-LUC) lacking NFAT-binding sites was also used (A). Pim-1 and β-actin protein levels were analyzed by Western blotting from the same cell lysates as in panels (A) and (D) (B, E).

When the PC12 cell lysates were subjected to Western blotting with anti-Pim-1 antibodies, we noticed that the endogenously expressed 44 and 33 kDa Pim-1 isoforms were barely detectable in unstimulated cells. By contrast, their steady-state levels were strongly upregulated by treatment with ionomycin and PMA (Fig. 2B). The Western blot analysis also confirmed that the expression levels of the ectopically expressed 33 kDa isoform of Pim-1 remained within the physiological range, while blotting of the same membranes with anti-β-actin antibodies verified that equivalent amounts of protein had been loaded to all lanes.

When we analyzed NFAT-luciferase activities 3 or 6 h after stimulation of cells with PMA and ionomycin and/or forskolin, we noticed that, unlike in T cells, forskolin was able to enhance NFATc-dependent transactivation even in the absence of PMA and ionomycin (Fig. 2C). Interestingly, the stimulatory effects of forskolin were much stronger at 6 h after induction, while the effects of Pim-1 were more striking already at the earlier 3 h time-point, when it was also able to potentiate the effects of forskolin. The slower upregulation of NFATc activity by forskolin correlated well with the delayed kinetics of the nuclear translocation of NFATc observed in the presence of forskolin (data not shown).

To further confirm that the effects of forskolin were mediated via enhanced calcineurin-dependent nuclear translocation of NFATc, we repeated part of the transactivation assays in the presence of CsA. Interestingly, although CsA was able to completely block the stimulatory effects of forskolin at the 3 h time-point, it only partially reduced the enhancing effects of Pim-1 observed in the presence of forskolin (Fig. 2D). When we again carried out a Western blot analysis from the cell lysates, we noticed that, unlike ionomycin and PMA, forskolin did not have any major effects on endogenous Pim-1 expression (Fig. 2E). By contrast, CsA administered together with forskolin appeared to slightly downregulate the steady-state levels of the ectopically expressed 33 kDa Pim-1 isoform, which may partially explain the weaker effects of Pim-1 observed in these cells.

### *3.3. Pim-1 enhances forskolin-induced catecholamine synthesis*

In response to NGF, differentiating PC12 cells start to produce and release the catecholamines dopamine (DA) and noradrenaline (NA). Therefore, we were interested in the potential effects of PMA, ionomycin and/or forskolin on the catecholamine contents of NGF-treated PC12 cells in the absence or presence of coexpressed Pim-1. While the levels of the catecholamine precursor L-DOPA were not significantly affected by a 3 or 6 h treatment with PMA and ionomycin, forskolin enhanced its formation in the presence of Pim-1 (Fig. 3A and data not shown). The cellular levels of the L-DOPA-derived products, DA and NA, were significantly reduced by PMA and ionomycin (Fig. 3B and data not shown), apparently due to enhanced secretion of these catecholamines, but not their precursor L-DOPA out of the cells. Again, Pim-1 increased DA and NA levels in forskolin-treated cells. By contrast, the kinase-deficient NT81 mutant of Pim-1 was not able to potentiate the effects of forskolin in any of the assays but rather inhibited catecholamine production even in unstimulated cells (see Appendix A). As an additional control, we analyzed the steady-state protein levels of tyrosine hydroxylase (TH), the rate-limiting enzyme in the synthesis of L-DOPA. However, TH levels were not significantly affected by Pim-1 expression or any of the employed treatments (Fig. 4A). Immunocytochemical analysis also confirmed that the intensity of the cytoplasmic staining of TH remained unchanged in all samples (Fig. 4B and data not shown).

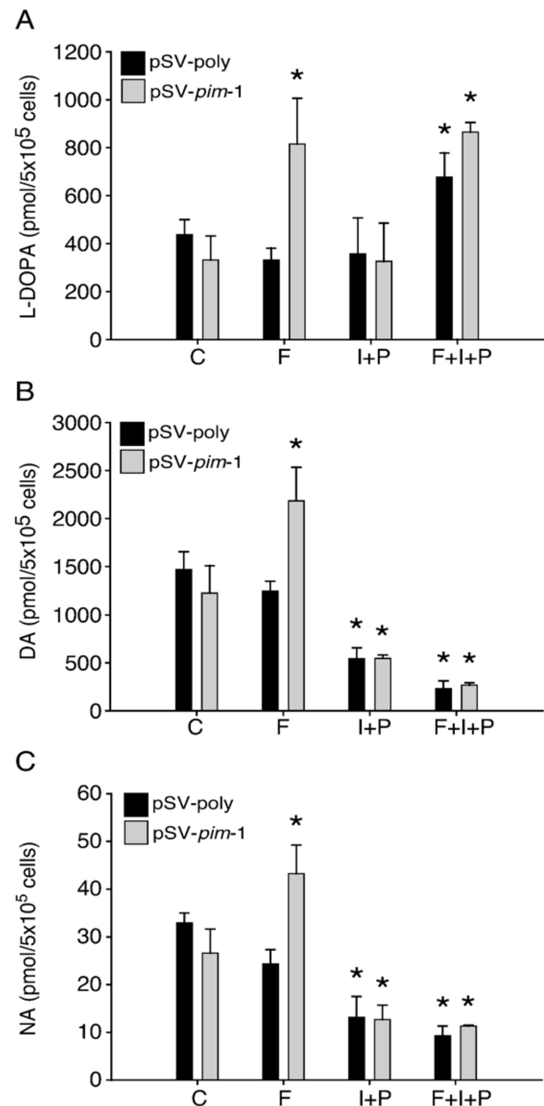


Fig. 3. Pim-1 enhances forskolin-induced catecholamine synthesis. L-DOPA (A), dopamine (DA; B) and noradrenaline (NA; C) contents were measured from transiently transfected PC12 cells treated for 3 h with indicated combinations of drugs (abbreviated as in Fig. 1).

## 4. Discussion

In this study, we show that NFATc-dependent transcription can be regulated very similarly in NGF-differentiated PC12 cells as in lymphoid cells [6,31], in undifferentiated PC12 cells [4] and in hippocampal neurons [12], even though the upstream signals and downstream target genes are likely to differ from each other in a cell-type-dependent fashion. The inducible NFATc activity can in turn be abolished by treatment with cyclosporin A, indicating tight regulation in a calcium- and calcineurin-dependent fashion. An interesting difference, however, is that in neuronal cells NFATc proteins can be persistently activated after only a brief calcium pulse [12], whereas sustained elevation of intracellular calcium concentrations is required in the nonexcitable lymphoid cells to maintain NFATc proteins in an active form in the nuclei [6,31]. In addition to previously reported expression of NFATc2 in PC12 cells [16,28], we now demonstrate that also the NFATc1 family member is expressed there. Furthermore, NFATc1 expression is observed also in neurons of hypothalamic neuroendocrine centers including paraventricular,

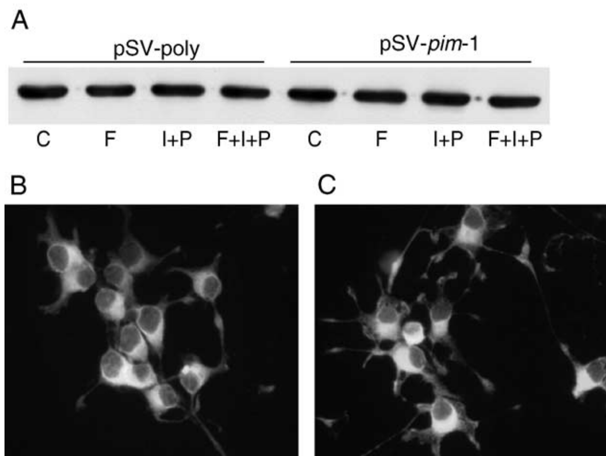


Fig. 4. Expression or subcellular distribution of tyrosine hydroxylase is not affected by Pim-1 or any of the drug treatments. (A) Transiently transfected PC12 cells were stimulated with indicated combinations of drugs (abbreviated as in Fig. 1) and analyzed for tyrosine hydroxylase protein levels by Western blotting. (B, C) Untransfected PC12 cells were left unstimulated (B) or were stimulated with all three drugs for 3 h (C), after which cells were fixed and cellular contents of tyrosine hydroxylase were determined by immunocytochemistry.

supraoptic, arcuat and suprachiasmatic nuclei (M. Glazova and P.J. Koskinen, unpublished data).

While the protein kinase A agonist forskolin stimulates NFATc-dependent transactivation in PC12 cells and inhibits it in T cells, we demonstrate that the Pim-1 kinase can enhance NFATc activity in both types of cells in a phosphorylation-dependent manner. While we have previously identified NFATc1 as a direct substrate for Pim-1 kinase in T cells [30], our current results indicate that Pim-1 can similarly target also other NFATc family members expressed in PC12 cells. Moreover, it is likely that the observed effects can be extended to other types of tissues which coexpress Pim and NFATc proteins. However, it should be noted that Pim-1 alone does not have any major effects on NFATc activity but only potentiates signaling pathways initiated by external stimuli. Therefore, net effects of Pim-1 vary depending on the cellular circumstances and appear to be most striking under suboptimal stimulation conditions, as recently also demonstrated with T cells of mice lacking all the three *pim* family genes [24].

Several recent reports have implicated NFATc proteins in neuronal development. They can be activated in primary neurons by neurotrophins, electrical activity or potassium depolarization [12,13,15] and may together with Pim-1 [18] be involved in regulation of synaptic plasticity and memory formation. Neurotrophins, such as the brain-derived neurotrophic factor, have critical roles in shaping neuronal connections during development [17] and mediate adaptive processes involved in cell survival, chronic pain, drug addiction, as well as learning and memory [11,19,41]. Interestingly, mice lacking functional calcineurin or NFATc2, c3 and c4 genes have dramatic defects in neurotrophin-induced axonal outgrowth, whereas neuronal differentiation or survival is not affected [13].

Differentiated PC12 cells share many characteristics with sympathetic neurons and are able to produce and release catecholamines, such as dopamine and noradrenaline. Our results indicate that catalytically active Pim-1 kinase can also modulate the neuroendocrine functions of PC12 cells. While the cellular catecholamine levels were dramatically decreased by PMA and ionomycin, the abundance of tyrosine hydroxylase protein remained unchanged. These results correlate well with the observed calcium-dependent enhancement of catecholamine secretion in PC12 cells by agents activating PKC or PKA [25,26,37]. Pim-1 slightly synergized with forskolin to stimulate production of the precursor L-DOPA as well as DA and NA. However, it should be noted that the effects of ectopically expressed Pim-1 in the catecholamine assays were strongly diluted by the presence of untransfected cells. In any case, it remains to be determined whether Pim-1 plays any direct role in catecholamine synthesis and/or secretion and whether this role is related to the ability of Pim-1 to enhance NFATc activity.

According to our Western blot analyses, the steady-state levels of the endogenously expressed 44 and 33 kDa Pim-1 isoforms are fairly low in PC12 cells, but their expression can be strongly upregulated by ionomycin and PMA. This may at least partially be due to the ability of these agents to enhance stability of Pim-1 protein, as previously shown in T cells [38]. By contrast, forskolin does not have any major effects on Pim-1 protein levels, even though it has been reported to induce *pim-1* mRNA expression in PC12 cells [10]. Since the levels of the ectopically expressed 33 kDa Pim-1 in unstimulated or in forskolin-treated cells were comparable to the endogenous levels observed in cells treated with ionomycin and PMA, this may also explain why exogenous Pim-1 affected catecholamine synthesis only in forskolin-treated cells and not in cells treated with ionomycin and PMA, where the Pim-1 levels were high also in untransfected cells.

PC12 cells as well as peripheral sympathetic neurons are able to express both IL-6 and IL-6 receptor mRNAs and produce IL-6 in an autocrine fashion [22,23]. Intriguingly, Pim-1 in PC12 cells is able to stimulate activation of, not only IL-2, but also IL-6 enhancer (M. Glazova and P.J. Koskinen, unpublished data), whereas IL-6 in hematopoietic cells has been shown to induce *pim*-1 expression [20]. Since Pim-1 acts as a survival factor for myeloid cells deprived of IL-3 [21] or subjected to genotoxic stress [27] and since autocrine production of IL-6 may be equally essential for the survival of neuronal cells [35], it would be interesting to determine whether Pim-1 could indeed act up- or downstream of IL-6 to protect neuronal cells from various types of apoptosis and whether NFATc proteins are also involved in regulation of IL-6 production, as previously described for other types of cells [1,36].

Taken together, our results indicate that the Pim-1 kinase can enhance NFATc activity in a phosphorylation-dependent manner and collaborate with agents activating the PKA pathway to modulate the neuroendocrine functions of the neuron-like PC12 cells and most likely also of primary neuronal cells.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molbrainres.2005.04.003.

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