Stimulation of *vgf* gene expression by NGF is mediated through multiple signal transduction pathways involving protein phosphorylation

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Abstract **The *vgf* gene encodes one of the most rapidly induced neuronal mRNAs identified in NGF-treated PC12 cells. Maximal inhibition of VGF mRNA induction was achieved using K-252a, an inhibitor of the NGF-receptor Trk tyrosine kinase, and by mutating both Y490 (SHC association site) and Y785 (PLC-γ1 association site) of Trk. Inhibitors of the NGF-activated protein kinase N (PKN) were found to partially and in some cases transiently block VGF induction by NGF while in PKA-deficient PC12 cells, VGF induction by NGF was comparable to that observed in parental PC12 cells. The binding of NGF to Trk therefore activates redundant signal transduction pathways which converge to regulate *vgf* gene expression.**

Key words: VGF; NGF; Trk; PC12 cell; Protein kinase N; Protein phosphorylation

1. Introduction

In the presence of nerve growth factor (NGF) and basic fibroblast growth factor (bFGF) but not epidermal growth factor (EGF), PC12 pheochromocytoma cells differentiate into sympathetic neuron-like cells [1]. Treatment of PC12 cells with NGF results in the rapid tyrosine phosphorylation of multiple cellular proteins [2] and the stimulation of several second messenger pathways through the actions of a number of tyrosine and serine/threonine kinases [3]. The importance of protein phosphorylation in the mechanism of action of NGF has been reinforced by the identification of the product of the *trk* tyrosine kinase protooncogene as the high affinity NGF receptor [4,5], and the demonstration that Trk is a necessary component of the NGF signaling pathway that is required for neurite outgrowth and the promotion of PC12 cell survival [6].

NGF-treatment stimulates $p21^{ras}$ GTP-binding activity, causing prolonged $p21^{ras}$ and ERK activation in comparison to EGF, a growth factor which does not cause neuronal differentiation of PC12 cells [7]. Selective inhibition of protein kinase activity through the application of purine analogs inhibits NGF-promoted neurite regeneration and the NGF-regulated serine/threonine protein kinase N (PKN) but does not block several other effects of NGF [8–10] suggesting that the development and maintenance of a neuronal phenotype results from the complex regulation of a number of protein kinases. Insight into the process of neuronal differentiation may be obtained through study of gene products such as VGF [11] which are rapidly activated more robustly by NGF than by EGF, in contrast to transcripts of the immediate early genes c-*fos*, c-*jun*, and *zif268* (NGFI-A), which appear to be induced similarly by either NGF, EGF, or serum stimulation [12]. In PC12 cells, VGF mRNA levels increase at least 15–30-fold after 3–6 h of NGF treatment and return to basal levels after approximately 48 h [13,14]. Induction of *vgf* gene expression is mimicked by activation of a Src-Ras-Raf cascade in PC12 cells [15], and is partially blocked by protein synthesis inhibitors [16,17] and downregulation of protein kinase C (PKC)-dependent pathways [14]. Here we utilize a number of protein kinase inhibitors, several PC12-derived cell lines that express wild type or mutant forms of Trk, and a PKA-deficient PC12 cell line to characterize the relative contributions of specific signal transduction pathways to the regulation of *vgf* gene expression by NGF.

2. Materials and methods

2.1. Cell culture

PC12 cells, PC12nnr5 cell lines expressing wild type and mutant Trks (generously provided by Dr. L.A. Greene), and PC12 variant A126–1B2 cells (kindly provided by Dr. J.A. Wagner) were grown on collagen-coated dishes [1]. Purine analogs were added to cultures from concentrated stocks as described [18]. Where specified, PC12 cells were treated with 2.5S NGF (Boehringer Mannheim), EGF (Collaborative), recombinant bovine bFGF (kindly provided by Dr. A. Baird, Whittier Institute), K-252a (generous gift of G. Guroff), MTA (Sigma), and ML-9 (Sigma). Following treatment, PC12 cells were rinsed three times with ice-cold phosphate-buffered saline and RNA was isolated as described below.

2.2. RNA isolation and RNase protection analysis

PC12 cells were lysed, nuclei and cytosol fractionated by centrifugation [19], and cytoplasmic RNA isolated [20]. Where indicated, total cellular RNA was isolated as described [21]. RNase protection analysis was performed [20] using antisense VGF exon 3 and cyclophilin probes [11] to quantify cytoplasmic VGF and cyclophilin (internal control) mRNA levels, respectively. Samples containing 10 μg of total RNA were analyzed in duplicate or triplicate, and protected RNA fragments were resolved on nondenaturing 5% polyacrylamide gels using *Msp*I-digested pBR322 DNA as molecular size markers. After autoradiographic exposure, bands were quantified by scintillation counting or

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Abbreviations: 2-AP, 2-aminopurine; bFGF, basic fibroblast growth factor; 6-MMPR, 6-methylmercaptopurine riboside; NGF, nerve growth factor; ODC, ornithine decarboxylase; PLC-γ1, phospholipase C-γ1, PKA, protein kinase A; PKC, protein kinase C; PKN, protein kinase N; 6-TG, 6-thioguanine.

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with a Molecular Dynamics Phosphoimager, and the results expressed as relative level of VGF mRNA, computed as the ratio of radioactivity of the treated sample to that of the control (treatment/control), with control defined as 1.

2.3. Northern blot analysis

RNA samples (10 μg per lane) were electrophoresed through 0.8% agarose gels containing 2.2 M formaldehyde, 40 mM morpholinepropanesulfonic acid (MOPS) buffer (pH 7), 10 mM sodium acetate and 1 mM EDTA, and then electrophoretically transferred to nylon membranes and hybridized to ^{32}P-labeled antisense RNA probes (10^6 cpm/ml hybridization buffer containing 5 × SSC, 1 × Denhardt's solution, 20 mM sodium phosphate (pH 6.8), 50% formamide, 0.5% SDS and 0.1 mg/ml denatured salmon sperm DNA) at 68°C for 16–24 h. RNA probes were synthesized from the following linearized cDNA clones: VGF8a [13] and pIB15 (cyclophilin) [22]. Blots were washed twice in 0.1 × SSC, 0.1% SDS for 20 min at 68°C, and then subjected to autoradiography.

2.4. Antibody preparation and Western blot analysis

Rabbit polyclonal antibodies were prepared against a VGF(amino acids 78–340)-trpE fusion protein. An IgG fraction was affinity-purified sequentially on trpE and VGF-trpE columns, essentially as described [23]. Crude cytoplasmic cell extracts were isolated by three rapid freeze thaw cycles followed by centrifugation at 16,000 × *g* for 15 min. Whole cell extracts were obtained following lysis in 2 × sample buffer [24]. Samples containing equal amounts of protein, and molecular size standards of 97.4, 66.2, 45, 31, 21.5 and 14.4 kDa (BRL) were co-electrophoresed on 10% SDS-polyacrylamide gels and then transferred to nitrocellulose. Protein transfer and loading were confirmed by staining filters with 0.2% Ponceau red in 3% trichloroacetic acid. Western blots were incubated with affinity purified anti-VGF IgG (1:1,000) and were developed using ECL reagents (Amersham).

3. Results and discussion

3.1. NGF and bFGF induce VGF mRNA levels in protein kinase A-deficient cells

Treatment of PC12 cells with cAMP analogs induces VGF mRNA levels by 2–3-fold [11,14] (Fig. 1, panel A). The presence of a CRE consensus sequence in the *vgf* promoter [11] prompted us to investigate whether there was a requirement for protein kinase A (PKA) in the induction of VGF mRNA by NGF and bFGF. We employed a PC12-derived cell line (A126–1B2) which is deficient in PKA activity [25]; in these cells the CRE-CAT fusion gene is not inducible by cAMP elevation [26]. As expected, treatment of A126–1B2 cells with N^6,2′-*O*-dibutyryladenosine 3′:5′-cyclic monophosphate (dbcAMP) did not induce VGF mRNA (Fig. 1, panel B). In contrast, treatment of these cells with NGF or bFGF resulted in a level of VGF mRNA induction which was comparable to that obtained in the parental PC12 cells (Fig. 1, compare panels A and B), suggesting that these growth factors induce *vgf* gene expression predominantly through PKA-independent pathways. Deletion analysis has suggested that two regions within the *vgf* promoter, overlapping the CRE and the CCAAT elements, are involved in NGF-induction [17,27]. It would therefore appear that distinct mechanisms and possibly different transactivating factors regulate *vgf* gene expression in cAMP-stimulated and NGF-treated PC12 cells. In fact, c-*fos* expression has been shown to be induced by growth factors and by cAMP analogs through pathways that interact with different regulatory elements [28].

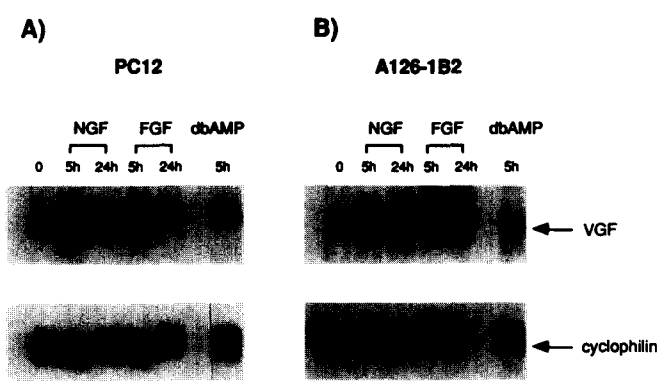

Fig. 1. NGF and bFGF induce VGF mRNA in protein kinase A-deficient cells. Parental PC12 cells (panel A) and A-kinase deficient PC12 derivative A126–1B2 cells (panel B) were incubated for the indicated lengths of time (h) with 100 ng/ml NGF, 100 ng/ml bFGF or 1 mM dbcAMP. Total RNA (10 μg/lane) was subjected to Northern analysis and the blot was hybridized to an antisense VGF riboprobe, autoradiographed, rehybridized to an antisense cycophilin riboprobe, and reautoradiographed. Portions of the autoradiograms are shown and the positions of the VGF and cyclophilin mRNAs are designated by the arrows.

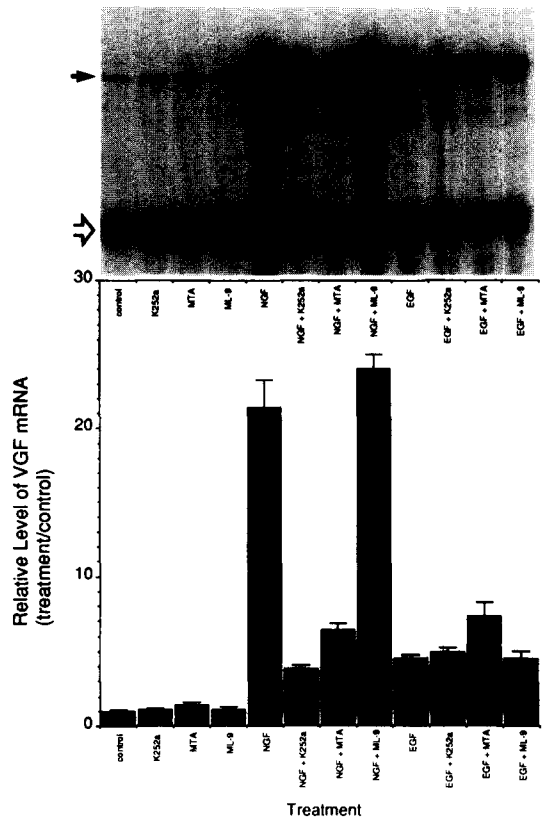

Fig. 2. K-252a and MTA block induction of *vgf* gene expression by NGF. PC12 cells were pretreated for 1 h in growth media alone (control) or in media supplemented with 200 μM K-252a, 3 mM MTA or 100 μM ML-9, after which the cultures were incubated for 3.25 h with no further additions or with either 100 ng/ml NGF or 3 ng/ml EGF added. RNase protection analysis was carried out and the bands corresponding to the fragments protected by the radiolabeled VGF (solid arrow) and cyclophilin (open arrow; internal control) antisense probes were quantified and results expressed as relative level of VGF mRNA. RNA was prepared from replicate plates and samples were assayed in duplicate; each bar is the mean ± S.E.M. ($n = 4$).

3.2. VGF mRNA induction by NGF is prevented by K-252a and the methylase inhibitor MTA

K-252a specifically blocks all of the biological actions of

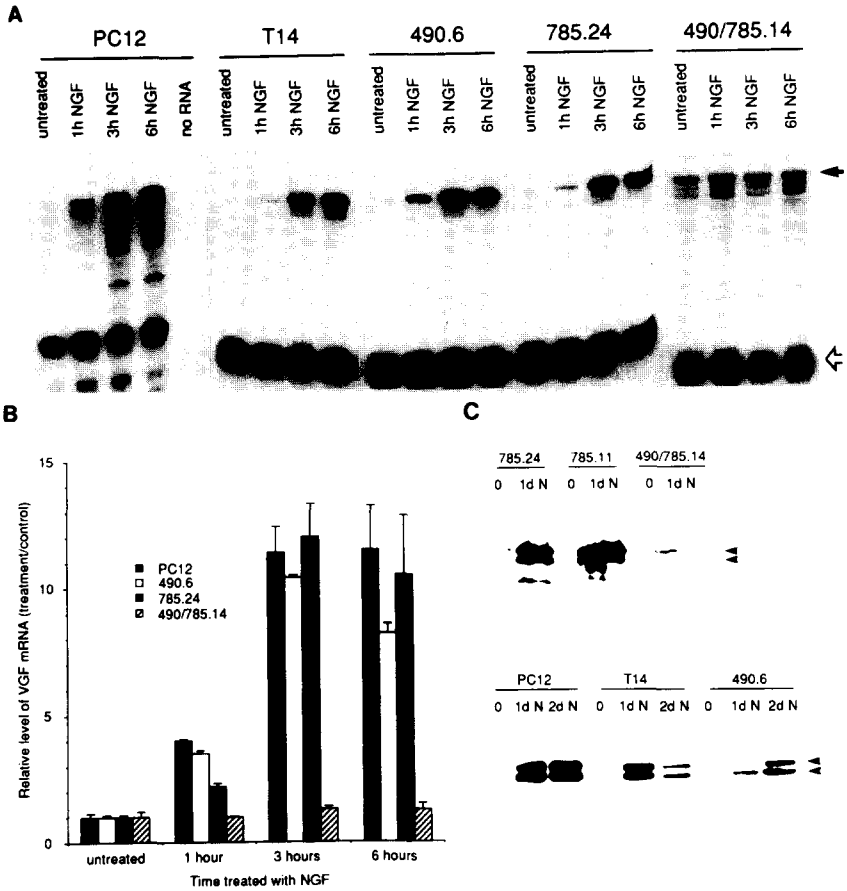

Fig. 3. Induction of VGF mRNA and polypeptide is blocked in NGF-treated PC12nnr5 cells expressing Trk Y490/Y785F but not in those expressing Trk Y490F or Trk Y785F. Untreated PC12 cells and PC12nnr5 cells, stably transfected with Trk (line T14) , Trk Y490F (line 490.6), Trk Y785F (line 785.24) or Trk Y490F/Y785F (line 490/785.14), or those treated with 50 ng/ml NGF for 1, 3, or 6 h, were subjected to RNase protection analysis. RNA samples were assayed in duplicate and fragments protected by the radiolabeled VGF (solid arrow) and cyclophilin (open arrow) antisense probes were quantified and the results expressed in panel B as relative level of VGF mRNA ± S.E.M. In panel C, samples containing equal amounts of protein, isolated from whole cell extracts of 785.24, 785.11 and 490/785.14 cells and crude cytoplasmic fractions of PC12, T14 and 490.6 cells were subjected to SDS-PAGE and Western blotting with anti-VGF IgG. After ECL development, blots were exposed to XAR-5 film for 5–10 s. The VGF polypeptide, a doublet migrating with an apparent size of ~85–90 kDa, is indicated by the arrowheads.

NGF but not other factors on PC12 cells [29] and inhibits the tyrosine kinase catalytic activity of Trk [30], the high affinity NGF-receptor [4,5]. PC12 cells were pretreated for 1 h with 200 μM K-252a and were then treated with either NGF or EGF and K-252a for 3.25 h. VGF mRNA levels, determined by RNase protection analysis [14], have been normalized to the unregulated mRNA cyclophilin to control for differences in input RNA amount and gel loading. K-252a treatment inhibited VGF induction in the presence of NGF by 86% (Fig. 2). The methylase inhibitor MTA, which blocks the neuronal differentiation of PC12 cells in the presence of NGF [19,31], reduced VGF mRNA induction by 74% when applied to NGF-treated cells (Fig. 2). For comparison, treatment with the myosin light chain kinase inhibitor ML-9 [32] had no significant effect on VGF mRNA induction by NGF, nor did any of the inhibitors affect the induction of VGF mRNA levels by EGF (Fig. 2).

3.3. *VGF induction following NGF-treatment is reduced in PC12nnr5 cells expressing Trk with mutations in both Y490 and Y785*

To determine the relative contributions of specific signal transduction pathways downstream of the NGF receptor to VGF induction, we examined regulation of VGF mRNA levels in PC12nnr5 cells transfected with wild type Trk or with Trk containing mutations (phenylalanine for tyrosine substitutions) in Y490, in Y785 or in both Y490 and Y785 [33]. PC12nnr5 cells lack Trk and fail to respond to NGF; expression of Trk restores NGF responsiveness in these cells [6,34]. The Y490 of Trk mediates association with SHC [33,35] while the Y785 of Trk is required for interaction with PLC-γ1 [33,36]. To control for possible differences in the kinetics or level of VGF mRNA induction in PC12nnr5-derived lines which might not be directly related to mutations in Trk, VGF mRNA levels in EGF-treated cells were also measured, and were found to be similar to EGF-treated PC12 cells (data not shown). As is shown in Fig. 3, panel A, mutation of either Y490 (line 490.6) or Y785 (line 785.24; similar results for line 785.11 not shown) did not substantially alter the magnitude or kinetics of VGF mRNA induction in NGF-treated cells compared to either T14 (PC12nnr5 cells transfected with Trk) or PC12 cells. VGF mRNA induction in NGF-treated cells expressing Trk with mutations in both Y490 and Y785 was not detectable (line 490/785.14; comparable results for 490/785.21 not shown). Quantitation of these results (panel B) is representative of three

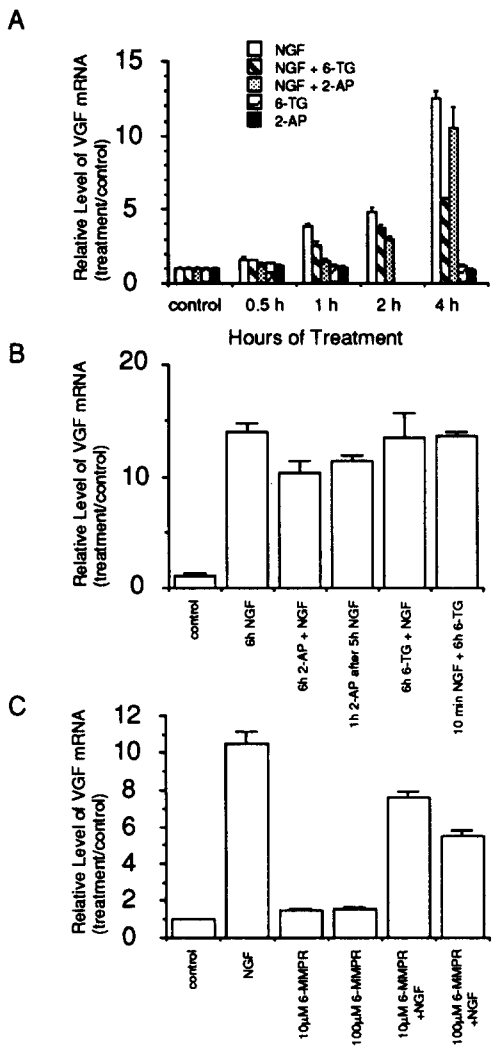

Fig. 4. Effect of treatment with 6-TG, 2-AP and 6-MMPR on the induction of VGF mRNA levels. VGF and cyclophilin mRNA levels were determined by RNase protection analysis and results expressed as relative level of VGF mRNA. Samples, containing 10 μg of total RNA [21], were assayed in triplicate; each bar is the mean ± S.E.M. In panel A, untreated PC12 cells (control) and those grown for 0.5, 1, 2, and 4 h in the presence of NGF, NGF + 6-TG, NGF + 2-AP, 6-TG or 2-AP were analyzed. In panel B, VGF mRNA levels were quantified in untreated PC12 cells (control) and cells treated for 6 h with NGF, 6 h with 2-AP + NGF, 1 h 2-AP + NGF following the previous addition of NGF for 5 h, 6 h with 6-TG + NGF, and for 10 min with NGF alone followed by the addition of 6-TG + NGF for 6 h. In panel C, VGF mRNA levels were quantified in untreated (control) and 5 h NGF-treated PC12 cells, all in the presence or absence of 10 or 100 μM 6-MMPR.

additional experiments; induction of VGF mRNA in T14 cells was not quantified because basal levels were below detection. Examination of VGF protein expression in untreated and NGF-treated cells by Western analysis confirmed these observations (panel C). VGF polypeptide induction in response to NGF-treatment was eliminated in the 490/785.14 line, but was still found in the 490.6, 785.11, and 785.24 lines, as well as in PC12 and T14 cells containing wild type Trk.

Regulation of *vgf* gene expression by NGF is therefore distinct from that of peripherin which is abolished by mutation of Y785 [37]. Since cells expressing Trk with the Y785 mutation contain tyrosine phosphorylated SHC and Grb2 in the absence of NGF [33] and have levels of VGF mRNA that are comparable to untreated PC12 cells, activation of SHC and Grb2 alone is not sufficient to trigger VGF mRNA induction. In cells expressing Trk Y490/Y785, in which both the PLC-γ1 and SHC association sites are mutated, VGF mRNA and polypeptide levels did not significantly change in response to NGF-treatment, indicating that activation of c-Ras, ERK 1 and possibly additional molecules is required for *vgf* gene induction by NGF. Since comparable induction of VGF mRNA levels was observed in each of the PC12, 490.6 and 785.24 cell lines, stimulation of the PLC-γ1 or the SHC pathway alone, or possibly additional signaling pathways that might be activated through association with either the PLC-γ1 or the SHC binding sites of Trk, is sufficient to maximally induce VGF expression.

3.4. *Partial inhibition of the induction of VGF mRNA in the presence of purine analogs*

Since purine analogs inhibit a number of the specific actions of NGF on PC12 cells, such as neurite regeneration and ornithine decarboxylase (ODC) induction, while differentially blocking others that include c-*fos*, c-*jun*, TIS1 and TIS11 induction [8, 9, 38, 39], we tested their effect on the levels of VGF mRNA in naive and NGF-treated cells. Purine analogs suppress several specific phosphorylation activities, and block in vitro activity of the NGF-stimulated kinase designated protein kinase N (PKN) at the concentrations employed [8,10,18,39]. As is shown in Fig. 4, panel A, 2-AP (10 mM) partially blocked the induction of VGF mRNA levels in PC12 cells treated with NGF for 1 and 2 h, but did not significantly inhibit induction after 4–6 h of NGF treatment ($P < 0.05$, one factor ANOVA, Scheffe F-test). Treatment with 6-TG (0.5 mM) resulted in a partial but significant block of VGF mRNA induction by NGF in cultures incubated for 1–4 h ($P < 0.05$, one factor ANOVA, Scheffe F-test). No significant difference in VGF mRNA levels was observed among cultures treated with NGF, NGF + 2-AP, and NGF + 6-TG for 6 h (Fig. 4, panels A and B). Prior analysis of these same RNA samples demonstrated that the induction of ODC by a 6 h treatment with NGF was blocked if the 2-AP was present for the last hour of NGF treatment [18]. Furthermore, a 10 min preincubation with NGF was sufficient to abolish the inhibition by 6-TG of NGF-dependent ODC induction [18]. Neither of these treatments were found to alter VGF mRNA levels (Fig. 4, panel B). In comparison to NGF treatment, treatment with NGF + 10 or 100 μM 6-MMPR led to significant 27% and 48% decreases, respectively, in VGF mRNA levels ($P < 0.05$, one factor ANOVA, Scheffe F-test) (Fig. 4, panel C). Induction of ODC mRNA by NGF, measured in these same RNA samples, was inhibited 86–93% by 6-MMPR [39]. Previous analysis revealed that the concentrations of 6-MMPR, 2-AP and 6-TG employed were sufficient to inhibit PKN kinase activity by 85–90% [8,18,39,40].

In addition to strongly inhibiting PKN, 2-AP inhibits Ca^{2+}/calmodulin-dependent kinase I and several other kinases including the ERK/MAP kinases [7,39,41,42], while reports differ as to whether 6-TG inhibits [7] or does not inhibit at all [41,42] ERK/MAP kinase activity. Finally, 6-MMPR appears to very selectively inhibit PKN but not MAP kinase activity [39] (C. Volonté, unpublished data). Partial blockade of VGF mRNA induction is unlikely to be due to partial inhibition of kinase activity since (i) these concentrations used under these condi-

tions have been shown to inhibit PKN activity [8,10,39] and (ii) some of these RNA samples have been previously analyzed and complete inhibition of ornithine decarboxylase induction by 2-AP and 6-MMPR was found [39,40]. Partial blockade by 2-AP and 6-TG of the induction of transin mRNA in NGF-treated PC12 cells has been observed, though time course studies were not performed to investigate whether the inhibitors delay rather than reduce transin mRNA induction [43]. In fact, induction of TIS1 (also designated NGFI-B or nur77) mRNA by NGF is delayed by 2-AP [38]. The finding that PKN is not activated by NGF in PC12 cells expressing dominant-negative v-Ras (C. Volonté and L.A. Greene, unpublished data) is consistent with a role for PKN in the regulation of VGF expression. PKN may be one component in the pathway that controls *vgf* gene induction, however other regulatory pathways must also contribute to this induction. In some cases, activation of these alternative pathways may lead to compensatory increases in VGF mRNA levels when PKN activity is blocked.

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