Nerve Growth Factor-Induced Differentiation of PC12 Cells Is Accompanied by Elevated Adenylyl Cyclase Activity

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Key Words

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Abstract

Rat pheochromocytoma (PC12) cells characteristically undergo differentiation when cultured with nerve growth factor (NGF). Here we show that NGF dramatically increased the adenylyl cyclase-activating property of forskolin in PC12 cells. This effect of NGF was well maintained even when NGF was removed after 4 days, even though the morphological features of neuronal differentiation were rapidly lost on removal of NGF. The enhanced cAMP production in response to forskolin could be due to a synergistic interaction between forskolin and endogenously released agonists acting on G_s-coupled receptors. However, responses to forskolin were not attenuated by antagonists of adenosine A2 receptors or pituitary adenylate cyclase-activating polypeptide (PACAP) receptors, suggesting that adenosine and PACAP were not involved. Adenylyl cyclases 3, 6 and 9 were the predominant isoforms expressed in PC12 cells, but we found no evidence for NGF-induced changes in expression levels of any of the 9 adenylyl cyclase isoforms, nor in the expression of G α_s . These findings highlight that NGF has a subtle influ-

ence on adenylyl cyclase activity in PC12 cells which may influence more than the neurite extension process classically associated with neuronal differentiation.

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Introduction

Neurotrophins, such as nerve growth factor (NGF), have been extensively studied for their ability to promote differentiation and survival of rat pheochromocytoma (PC12) cells [1]. NGF treatment causes differentiation of PC12 cells resulting in growth arrest accompanied by elongation of neurites [2]. Although NGF promotes neurite extension through a cAMP-independent signaling pathway involving Ras, protein kinase C and extracellular signal-regulated protein kinase [3], agents which elevate cAMP levels have effects alone and can exhibit a synergistic effect with NGF on neurite outgrowth [4–6]. Pituitary adenylate cyclase-activating polypeptide (PACAP) regulates neuronal differentiation through a cAMP-dependent mechanism [7, 8], and adenosine, acting via A2a receptors (A2aR), also produces cAMP-dependent phenotypic changes in PC12 cells [9].

The cyclooxygenase-1 enzyme responsible for prostaglandin production behaves as a delayed response gene in PC12 cells exposed to NGF [6, 10]. During studies to assess the role of prostaglandins during PC12 cell differentiation [11], we noted a marked increase in forskolin-stimulated adenylyl cyclase (AC) activity over a 6-day treatment period with NGF. Forskolin directly activates AC and can amplify cAMP-dependent signaling activated by G_s -coupled receptors [12]. Thus, G_s -promoted enhancement of AC activity in response to forskolin occurs not only when cells are incubated with exogenously administered agonists for G_s -coupled receptors but also by agonists that can be endogenously released by cells. Many cells are capable of releasing AMP which can undergo extracellular degradation to adenosine via ectonucleotidases [13]. Indeed, endogenously released adenosine has been shown to act synergistically with forskolin to enhance cAMP production through A2aR in PC12 cells [14]. Furthermore, NGF can increase PACAP production by PC12 cells [15] which could stimulate PACAP-specific receptors (PAC1R) to generate cAMP and inositol phosphates by coupling to G_s and G_q proteins, respectively [16]. Therefore, if NGF caused the increased expression of components of G_s -dependent cell signaling pathways (e.g., G_s -coupled receptors and/or their endogenous ligands), then forskolin could synergize with these factors and generate the responses we observed. Cyclic AMP-dependent protein kinases have an essential role in neuronal differentiation and synaptic plasticity [17–19], and modulation of cAMP-dependent signaling may be crucial in developing methodologies to generate pure populations of neurons from human neural stem cells [20]. Therefore, the aim of this study was to identify which factor(s) were responsible for the enhanced responses to forskolin observed in our PC12 cell line following incubation with NGF and to determine the relationship between increased AC activity and the process of neuronal differentiation. Having previously shown that NGF increased A2aR and PACAP mRNA expression in PC12 cells [6], we initially focused on these factors in addition to other components of cAMP-dependent cell signaling pathways in PC12 cells.

Materials and Methods

Materials

Mouse nerve growth factor (mNGF 2.5s) was purchased from Alomone Labs (Jerusalem, Israel). [3 H]adenine was purchased from GE Healthcare (Hong Kong). TRIzol, DNase I (Amp Grade), oligo-d(T)₂₀ primer, dNTPs and M-MVL were from Invitrogen

(Carlsbad, Calif., USA), and FastStart TaqDNA Polymerase was from Roche Diagnostics (Mannheim, Germany). GelRed™ was purchased from Biotium (Hayward, Calif., USA). PACAP-(1–38) and PACAP-(6–38) were purchased from Phoenix Pharmaceuticals (Beijing, China), and ZM241385 was purchased from Tocris Bioscience (Bristol, UK). All other compounds were supplied by Invitrogen or Sigma Chemical Co. (St. Louis, Mo., USA).

Cell Culture

PC12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with heat-inactivated horse serum (HIHS: 10%, v/v), fetal bovine serum (5%, v/v), penicillin (100 U/ml) and streptomycin (100 µg/ml). Cells were routinely grown on 100-mm tissue culture dishes at 37°C in a humidified atmosphere of 5% CO₂/95% air, and medium was changed every 3 days. Undifferentiated cells were plated in collagen-coated (0.2 mg/ml) tissue culture plates for assay. To study the effect of NGF, cells were cultured for 1 day in collagen-coated assay plates, then washed and incubated in low serum medium (DMEM containing 1% HIHS). NGF (50 ng/ml) or an equivalent volume of DMEM was then added (taken as day 0). Medium $\pm$ NGF was replaced every 2 days.

cAMP Assay

AC activity was assayed in Hepes-buffered saline containing 1 mM 3-isobutyl-1-methylxanthine (IBMX) to inhibit cyclic nucleotide phosphodiesterase activity, as previously described [21]. Briefly, PC12 cells (3×10^5 cells/well) were cultured in 0.5 ml medium in 24-well plates, and incubated with [3 H]adenine (2 µCi/ml; 1 µCi/well) for 24 h prior to assay. The production of [3 H]cAMP from cellular [3 H]ATP was estimated as the ratio of radiolabeled cAMP to total AXP (i.e. cAMP, ADP and ATP), and is expressed as $[\text{cAMP}]/[\text{total AXP}] \times 100$ (i.e. % conversion). All assays were performed in duplicate. Cells were preincubated with antagonists/inhibitors for 30 min as appropriate and then incubated with forskolin (1 µM) or receptor agonists for 30 min.

Morphometric Analysis

PC12 cells (5×10^4 cells/well) were cultured in 2 ml medium in 6-well plates. At specific time points, phase-contrast images were captured (DS Camera Control Unit DS-L1, Nikon) under 100 $\times$ magnification from at least 5 views per well. The images were quantified using Scion Image with the observer unaware of the treatment group. One hundred cells per well were scored for differentiation defined as (i) the proportion of cells with neurite/s equal to or greater than the diameter of the cell body, (ii) the number of neurites per cell, and (iii) the length of longest neurite.

RT-PCR

Total RNA was extracted with TRIzol and treated with DNase I to eliminate any contaminating genomic DNA. First-strand complementary DNA was synthesized using the M-MLV reverse transcriptase system followed by FastStart TaqDNA Polymerase for PCR amplification. The primers for AC1–9 and GAPDH were taken from published sequences [22]. Primer sequences for A2aR, A2bR, $G\alpha_s$ were from Castillo et al. [23] and those for PACAP and PAC1R from Hashimoto et al. [15] and Braas and May [24], respectively. PCR was initiated by a 5-min denaturation at 95°C, followed by 34 cycles (for AC3, AC6, AC7, AC9, A2bR, PACAP, PAC1R and $G\alpha_s$ and GAPDH) or 40 cycles (for AC1, AC2, AC4,

Fig. 1. NGF enhances forskolin-stimulated AC activity in PC12 cells. PC12 cells were cultured in **a** low serum medium or **b** low serum medium + NGF (50 ng/ml) for up to 6 days, with replacement of medium every 2 days. ○ = Basal activity in control cells, ● = forskolin (1 μM)-stimulated control cells, □ = basal activity

in NGF-treated cells, ■ = forskolin (1 μM)-stimulated NGF-treated cells. Results are mean ± SEM, n = 3. * p < 0.05, *** p < 0.001 compared with responses at day 0. Forskolin-stimulated responses were significantly increased in control cells (p < 0.05) and in NGF-treated cells (p < 0.001); analyzed by two-way ANOVA.

AC5, AC8 and A2bR) of 30 s at 54°C and 50 s at 72°C, and terminated by a final extension for 7 min at 72°C. The PCR products were resolved using a 1.5% agarose gel containing GelRed™, and the DNA bands were visualized by UV illumination and were quantified by Quantity One software (Bio-Rad Laboratories, Hercules, Calif., USA). The relationship between the amount of PCR products and the number of amplification cycles (27–36 for A2aR and Gα_s; 27–39 for PACAP; 30–39 for AC1, 6, 7, 9, GAPDH and PAC1R; 36–42 for AC1, 2, 4, 5, 8 and A2bR) was linear (data not shown).

Data Analysis

Values reported are means ± SEM. Comparisons between groups were made using ANOVA with Bonferroni's post-tests. All analyses were performed using GraphPad Prism software version 5.1 (GraphPad Software Inc., San Diego, Calif., USA). Statistical significance was taken as p < 0.05.

Results

NGF Increases AC Activity in PC12 Cells

Maintaining PC12 cells in low serum medium (1% HIHS) produced a small, but statistically significant, increase in both basal and forskolin-stimulated AC activity after 6 days (fig. 1a). When PC12 cells were incubated in low serum medium with NGF (50 ng/ml), there was a much more dramatic increase in forskolin-stimulated

[³H]cAMP production after 4 days (fig. 1b). To determine if the changes in AC activity were caused by gene changes early in the differentiation process, PC12 cells were incubated with NGF for the first 2, 4 or 6 days, and AC activity assayed on day 6. Withdrawal of NGF after 2 days generated final responses similar to non-NGF-treated cells, but forskolin-stimulated responses were maintained on day 6 even after the withdrawal of NGF after 4 days (fig. 2).

Classical markers of neuronal differentiation (proportion of cells expressing neurites, number of neurites per cell, and length of longest neurite) would be expected to show similar profiles on NGF withdrawal if the NGF induced differentiation and changes in AC activity were related. Results in figure 3 show that withdrawal of NGF from PC12 cells after both 2 and 4 days of treatment produced a significant loss of morphological features associated with differentiation by day 6.

To determine if NGF had a direct effect on AC activity, PC12 cells were incubated for 6 days (± NGF), washed as usual, then the effect of NGF (50 ng/ml) was tested in the presence or absence of forskolin (1 μM). NGF had no effect on AC activity alone in any of the 3 treatment groups tested: control cells day 0, control cells day 6, and NGF-treated cells day 6 (fig. 4). Similarly, NGF did not acutely enhance forskolin-stimulated responses.

Fig. 2. Effect of withdrawal of NGF on AC activity in PC12 cells. PC12 cells were cultured in low serum medium $\pm$ NGF (50 ng/ml) for up to 6 days, with replacement of medium every 2 days. After 2 or 4 days' treatment with NGF, the NGF was removed from the culture conditions. **a** Basal and **b** forskolin (1 μ M)-stimulated [3 H]cAMP production. $\circ$ = Control cells, $\bullet$ = NGF 50 ng/ml-

treated cells, $\square$ = cells treated with NGF for 2 days only, $\triangle$ = cells treated with NGF for 4 days only. Results are mean $\pm$ SEM, $n = 3$. ** $p < 0.01$, *** $p < 0.001$ compared with control group. ## $p < 0.01$, ### $p < 0.001$ compared with 6-day NGF group (analyzed by two-way ANOVA).

Fig. 3. Markers of neuronal differentiation decrease on withdrawal of NGF from PC12 cells. PC12 cells were cultured in low serum medium $\pm$ NGF (50 ng/ml) for up to 6 days, with replacement of medium every 2 days. After 2 or 4 days' treatment with NGF, the NGF was removed from the culture conditions. **a** Proportion of cells with neurites defined as greater than one cell body in diameter, **b** number of neurites per cell, and **c** length of the longest neurite on neurite-bearing cells. $\circ$ = Control cells, $\bullet$ = NGF 50 ng/ml-treated cells, $\square$ = cells treated with NGF for 2 days only, $\triangle$ = cells treated with NGF for 4 days only. Data are mean $\pm$ SEM of 6 wells of cells from 2 independent experiments, with at least 100 cells analyzed per well. *** $p < 0.001$ compared with control group. ## $p < 0.01$, ### $p < 0.001$ compared with 6-day NGF group (analyzed by two-way ANOVA).

Fig. 4. NGF has no direct effect on AC activity in PC12 cells. PC12 cells were cultured in low serum medium and assayed for [3 H]cAMP production in response to assay buffer (Con) or NGF (50 ng/ml) $\pm$ forskolin (Fors, 1 μ M) on day 0 (□) and day 6 (▨), and on cells cultured with NGF (50 ng/ml) for 6 days (■). Results are mean and SEM, $n = 3$.

NGF Does Not Alter AC Isoform Expression

Because of the lack of antibodies specific to AC isoforms [25], we used semiquantitative RT-PCR to assess the expression patterns of all 9 AC isoforms (see fig. 5a). AC3, AC6 and AC9 showed the highest expression, and AC7 was moderately expressed. AC1, AC2, AC4, AC5 and AC8 were much less well expressed and required 6 more cycles of PCR amplification for reasonable detection. There were no significant changes in AC mRNA expression patterns in PC12 cells after incubation for 6 days with NGF (fig. 5b).

A more detailed investigation was made over the 6-day treatment period which revealed no significant changes in the expression of AC1–9 mRNA when determined on days 0, 2, 4 and 6 in the presence or absence of NGF (fig. 6).

NGF Does Not Influence Adenosine Receptor or PACAP Receptor-Dependent Signaling in PC12 Cells

The marked increase in response of NGF-treated PC12 cells to forskolin could be due to synergistic activation of AC by coactivation of G_s -coupled A2aR [14]. The A2 receptor agonist CGS21680 stimulated [3 H]cAMP production in both control and NGF-treated PC12 cells by approximately 8-fold, and the A2 receptor antagonist ZM241385 inhibited this response by 89% (fig. 7). Any

Fig. 5. Relative expression levels of AC isoforms in PC12 cells. PC12 cells were cultured for 6 days in low serum medium in the presence or absence of NGF (50 ng/ml). mRNA expression was detected using 34 cycles of RT-PCR for AC3, 6, 7 and 9, and 40 amplification cycles for AC1, 2, 4, 5 and 8. **a** PCR products are shown on an agarose gel. Expected product sizes are AC1 446 bp (1), AC2 334 bp (2), AC3 456 bp (3), AC4 283 bp (4), AC5 456 bp (5), AC6 495 bp (6), AC7 256 bp (7), AC8 478 bp (8), AC9 360 bp (9), GAPDH 317 bp (G). **b** AC mRNA of control cells (□) and cells treated with NGF (50 ng/ml (■)) for 6 days. Semiquantitative RT-PCR indicates a lack of effect of NGF after 6 days. Results are mean and SEM, $n = 3$ ($p > 0.05$; analyzed by two-way ANOVA).

potential A2aR blocking activity of IBMX used in the AC assay did not prevent the [3 H]cAMP elevating properties CGS21680. The response to forskolin in the presence of an A2 receptor agonist was greater than an additive response of the 2 individual agents. For example, in NGF-treated cells, the response to CGS21680 + forskolin was 28-fold control (basal) activity compared to 8-fold for forskolin alone. A comparison of agonist-alone-stimulated responses showed a significant effect of NGF treatment ($p < 0.001$, two-way ANOVA). Adenosine deaminase (ADA) was used to eliminate endogenous adenosine, but neither ZM241385 nor ADA had any effect on forskolin-stimulated [3 H]cAMP in control or NGF-treated cells. ZM241385 inhibited the response to CGS21680 + forskolin, but only down to the response size expected of forskolin alone.

Fig. 6. NGF has no effect on AC isoform expression over 6 days. Expression of AC1–9 mRNA was analyzed by semiquantitative RT-PCR and is expressed relative to GAPDH mRNA expression in the control group on day 0. Data shown for AC3, AC6 and AC9 mRNA only. ○ = Control cells, ● = NGF 50 ng/ml-treated cells. Results are mean and SEM, n = 3.

Fig. 7. The enhanced AC response to forskolin is not due to endogenously produced adenosine. PC12 cells were cultured for 4 days in **a** low serum medium or **b** low serum medium + NGF (50 ng/ml) and assayed for [3H]cAMP production in response to assay

buffer (Con) or CGS21680 (CGS, 1 μM) ± forskolin (Fors, 1 μM). Control cells (□), cells pretreated with ZM241385 (1 μM) (■) or adenosine deaminase (1 U/ml) (▨). Results are mean and SEM, n = 3. *** p < 0.001.

Fig. 8. The enhanced AC response to forskolin is not due to endogenously produced PACAP. PC12 cells were cultured for 6 days in **a** low serum medium or **b** low serum medium + NGF (50 ng/ml) and assayed for [3 H]cAMP production in response to assay

buffer (Con) or PACAP-(1–38) (PACAP, 1 nM) $\pm$ forskolin (Fors, 1 μ M). Control cells (□) and cells pretreated with PACAP-(6–38) (1 μ M) (■). Results are mean and SEM, $n = 3$.

The marked increase in response of NGF-treated PC12 cells to forskolin might alternatively be due to endogenously produced PACAP [15]. The PAC1R agonist PACAP-(1–38) [26] stimulated [3 H]cAMP production in both control and NGF-treated PC12 cells by approximately 16-fold, and the PAC1R antagonist PACAP-(6–38) inhibited this response by 63% (fig. 8). The response to forskolin in the presence of a PAC1R agonist was greater than an additive response of the 2 individual agents. For example, in NGF-treated cells, the response to PACAP-(1–38) + forskolin was approximately 70-fold control (basal) compared to 5-fold for forskolin alone. A comparison of agonist-alone-stimulated responses showed a significant effect of NGF treatment ($p < 0.01$, two-way ANOVA). PACAP-(6–38) had no effect on forskolin-stimulated [3 H]cAMP in control or NGF-treated cells and produced a modest decrease in activity in cells stimulated with both PACAP-(1–38) and forskolin.

NGF Does Not Alter Expression of G_s -Coupled Receptors or $G\alpha_s$ Protein

To compliment the AC assays, we looked for changes in the expression of G_s -coupled adenosine and PACAP receptor mRNA following NGF treatment of PC12 cells. Both adenosine A2a and A2b receptors can stimulate AC through G_s proteins [13], but mRNA levels for these receptors were not significantly changed in NGF-treated cells (fig. 9). Furthermore, the expression of the endogenous agonist PACAP and its target receptor PAC1R were

also unaffected by NGF treatment (fig. 9). Any changes in $G\alpha_s$ expression would be expected to facilitate forskolin-stimulated responses in the presence of an activated G_s -coupled receptor. Therefore, we determined if NGF had any effect on the expression of $G\alpha_s$ mRNA. As seen in figure 9, NGF produced no obvious change in $G\alpha_s$ mRNA expression.

Discussion

Although NGF induces neuronal differentiation in a cAMP-independent manner [3], it is clear that NGF and cAMP appear to have independent but complimentary activities in terms of stimulating neurite outgrowth by PC12 cells [4–6]. We show herein, however, that NGF additionally appears to sensitize the AC/cAMP cell signaling pathway, resulting in a dramatic facilitation in forskolin-stimulated activation of AC after 4 days of treatment. Overexpression of protein kinase A in a hippocampal progenitor cell line has been shown to enhance neurite outgrowth [27], therefore, this sensitization of the AC/cAMP cell signaling pathway by NGF would be predicted to enhance the process of neuronal differentiation. Facilitation of forskolin-stimulated AC activity was reversible after 2 days' treatment of PC12 cells with NGF, but was well maintained when NGF was withdrawn after 4 days. As expected from Greene and Tischler [2], classical morphometric indices of neuronal differentiation were

Fig. 9. NGF does not change the expression of adenosine or PACAP receptors, nor the expression of $G\alpha_s$. PC12 cells were cultured for 6 days in low serum medium in the presence or absence of NGF (50 ng/ml). mRNA expression was detected using 40 cycles of RT-PCR for A2bR and 34 cycles for A2aR, PACAP, PAC1R and $G\alpha_s$. **a** PCR products are shown from the same agarose gel. Expected product sizes are PACAP 321 bp (lane 2), PAC1R 449 bp (lane 3), A2aR 150 bp (lane 4), A2bR 160 bp (lane 5), $G\alpha_s$ 770 bp (lane 6), and GAPDH 317 bp (lane 1). **b** AC mRNA of control cells (□) and cells treated with NGF (50 ng/ml (■)) for 6 days. Semi-quantitative RT-PCR indicates a lack of effect of NGF. Results are mean and SEM, $n = 3$ ($p > 0.05$; analyzed by two-way ANOVA).

much less stable and decreased towards the level seen in non-NGF-treated cells once NGF was removed. Changes in markers of neural differentiation do not necessarily parallel changes in neurite outgrowth [28, 29], and indeed there appears to be a dissociation between NGF-induced sensitization of AC/cAMP cell signaling and the process underlying neurite extension.

The most commonly studied G_s -coupled receptors in PC12 cells are those responding to adenosine (A2aR) and PACAP (PAC1R), with both agonists regulating neuronal differentiation through a cAMP-dependent mechanism [7–9]. Forskolin can amplify cAMP-dependent signaling involving activated $G\alpha_s$ [12], and the extent of forskolin-stimulated cAMP production in PC12 cells is reported to be dependent on endogenously-produced adenosine acting on A2aR [14]. Therefore, because we had shown that

NGF increased A2aR and PACAP mRNA expression in PC12 cells within 12 h of treatment [6], we first focused on determining if NGF had altered adenosine/A2aR or PACAP/PAC1R-dependent cell signaling.

When PC12 cells were cultured in low serum medium with NGF for 4–6 days, [3 H]cAMP production in response to CGS21680 and PACAP-(1–38) was increased, with both responses being inhibited by their respective receptor antagonists ZM241385 and PACAP-(6–38). However, the response to forskolin alone was not attenuated by either ZM241385 or PACAP-(6–38), or by ADA to eliminate endogenous adenosine. Therefore, although the enhanced sensitivity of AC signaling was not restricted to just forskolin-stimulated responses, it did not depend on any increase in adenosine or PACAP.

Long-term exposure of PC12 cells to adenosine might be expected to decrease A2aR activity [30], but we found no such evidence. Furthermore, although we saw enhanced [3 H]cAMP production in response to CGS21680, A2aR or A2bR mRNA expression was unchanged in NGF-treated cells. NGF has been reported to transiently increase PACAP mRNA expression in PC12 cells [15] and PACAP responsiveness [16] in NGF-treated PC12 cells, with a time scale matching our observed changes in responsiveness to forskolin following NGF treatment. However, although we see some evidence of increased [3 H]cAMP production in response to PACAP-(1–38) after NGF treatment, we did not see any increase in PACAP or PAC1R mRNA expression. Taken together, these results cannot support the hypothesis that adenosine or PACAP-dependent cell signaling mechanisms are involved in our observed responses of PC12 cells to NGF, but do not preclude activation of another G_s -coupled receptor system not identified in our initial gene profile study [6].

Another option is that NGF directly activates AC via a $G\alpha_s$ -dependent pathway, and that this results in the enhanced signaling of forskolin in NGF-treated cells. Although it has been reported that NGF can directly increase cAMP in PC12 cells [31], possibly via a ‘soluble’ rather than a ‘transmembrane’ AC [32], we and others [7] failed to observe such an effect. It is noticeable so far that PC12 cells in different laboratories produce different responses, not only for adenosine [9] but also for PACAP [33]. Therefore, clonal variations of PC12 cell lines are likely responsible for the different cAMP responses of PC12 cells to A2aR agonists and to NGF which we have observed. Nevertheless, given the acknowledged role of cAMP in neuronal differentiation, the responses observed in our PC12 cell clone are worth pursuing.

The increased responses to forskolin in NGF-treated PC12 cells could reflect changes downstream of adenosine and PACAP receptors. AC, rather than G_{α_s} , is considered to be the rate-limiting step distal to G-protein-coupled receptors which limits agonist-mediated increases in cAMP production [34], and clearly the relative expression of different AC isoforms would ultimately help determine the fate of the cell. The 'transmembrane' AC isoforms can be divided into 4 groups: Group I includes isoforms 1, 3 and 8 which are stimulated by calcium/calmodulin; Group II includes isoforms 2, 4 and 7 which are stimulated by $G\beta\gamma$ subunits; Group III includes isoforms 5 and 6 which are distinguished by their sensitivity to inhibition by Ca^{2+} and $G\alpha_i$ isoforms, and Group IV includes isoform 9 which is highly insensitive to forskolin [35]. Because of the lack of antibodies specific to AC isoforms [25], we used semiquantitative RT-PCR to assess the expression patterns of all 9 AC isoforms. We detected the mRNA expression of all 9 AC isoforms, with AC3, AC6 and AC9 being particularly abundant. AC3 and AC6 have been noted previously in PC12 cells [36, 37], and as AC9 is nonresponsive to forskolin, AC3 and AC6 may be of importance in our study. AC6 is thought to participate in neuronal signal integration [38], and differential regulation of AC isoforms has been identified as a crucial factor in controlling the differentiation of P19 teratocarcinoma cells [39], controlling the dedifferentiation of vascular smooth muscle cells [40], and in enhancing cAMP-mediated cytoskeletal reorganization [41]. Because the catalytic activity of AC6 is relatively low compared to other AC isoforms such as AC2, any upregulation of AC6 expression might lower the basal cAMP level which in turn allows for stronger activation by G_{α_s} -mediated interactions or by forskolin [42]. Although we did not detect any NGF-dependent changes in AC isoform expression over 6 days, it remains possible that changes at the protein level may have occurred.

Although cAMP may have a role in facilitating neurite maturation, it is thought that G_{α_o} is the principal G protein in stimulating neurite outgrowth [43]. Indeed, $G_{\alpha_{o1}}$ increased following NGF treatment of PC12 cells, while G_{α_s} did not [44, 45]. When we examined the expression of G_{α_s} after 6 days' treatment of PC12 cells with NGF, we also failed to detect any significant changes. Because a phosphodiesterase inhibitor is included in the AC assay, it is unlikely that facilitated cAMP signaling is due to down-regulated phosphodiesterase activity. Therefore, having discarded the possibility that the enhanced sensitivity of AC in NGF-treated PC12 cells results from changes in A2aR, PAC1R, AC isoforms, G_{α_s} or phosphodiesterases,

we are left with some other options not addressed in this current study. For example, the enhanced AC activity observed in our PC12 cell line might yet be due to an unidentified G_s -coupled receptor activated by an endogenously produced ligand, or due to NGF-induced downregulation of a G_i -dependent cell signaling pathway.

To speculate further, PACAP induces neurite outgrowth by induction of translocation of PAC1R into calveolin-enriched microdomains, leading to stronger PAC1R-AC interactions and elevated cAMP production [46]. Interestingly, it is AC6 which colocalizes in these calveolin-rich microdomains [46], and AC6 is one of the major AC isoforms in PC12 cells. The consequences of any AC6-dependent changes are complex. Recently, studies have identified crosstalk between tyrosine kinase receptors and G-protein-coupled receptors [47], with AC6 activity being enhanced by tyrosine kinase-mediated serine phosphorylation [48]. Thus, NGF could directly sensitize AC6-dependent signaling and regulate cAMP-mediated responses. In addition, AC is thought to have a role beyond simply responding to G-protein activation and the coupling of a particular isoform of AC to functions within a single cell type [47]. There are now emerging roles of AC interacting proteins other than G-proteins [49–51], which need to be considered, opening up even cAMP-independent roles for AC6 in neuronal cells. For example, Snapin (a binding protein of SNAP25 which is a component of the SNARE complex) is an interacting protein for AC6 which is thought to modulate neurotransmitter release [50].

Although all these aspects of AC6-dependent signaling in PC12 cells are of interest, the clonal variation in PC12 cell responsiveness complicates the widespread application of the conclusions. However, cAMP is crucial in facilitating the generation of dopaminergic neurons from neuronal stem cells [52–54], and understanding this process will be essential to the therapeutic application of stem cells in treating Parkinson's disease. Therefore, future studies should consider how neurotrophins might facilitate AC/cAMP-mediated transformation of neuronal stem cells to a neuronal phenotype with the characteristics of dopaminergic neurons.

In conclusion, we have clearly demonstrated that changes occur in AC/cAMP-dependent cell signaling pathways when PC12 cells are cultured in low serum conditions, and that these effects are markedly increased by the presence of NGF. In particular, there is a dramatic increase in the responsiveness of PC12 cells to the cAMP-elevating properties of forskolin. In this report, we show that NGF does not appear to enhance forskolin-stimulat-

ed AC activity by increasing the production of adenosine or PACAP, or by affecting expression of A2a, A2b or PAC1R. Neither does NGF appear to alter the expression levels of AC1–9 or of G_{α_s} . Therefore, the results of our current study indicate that NGF may have a more subtle influence on AC activity, and the precise role of this change in directing neuronal differentiation remains to be determined.

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