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Nerve growth factor inhibits PC12 cell PDE 2 phosphodiesterase activity and increases PDE 2 binding to phosphoproteins

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Abstract

Nerve growth factor (NGF) has been shown to increase cyclic AMP in PC12 cells and to potentiate the actions of other agents that raise cyclic AMP. In our studies, NGF causes over 50% loss of PDE 2 activity (cyclic GMP-stimulated cyclic nucleotide phosphodiesterase) in PC12 cells within 24 h. After 72 h of NGF treatment, cyclic AMP hydrolysis in PC12 extracts is no longer cyclic GMP-stimulated. NGF deprivation increases the phosphodiesterase activity of treated cells. NGF does not decrease either PDE 2 mRNA or immunoreactivity of PDE 2A2 protein. Incubation of whole cells with micromolar Na_3VO_4 mimics NGF treatment, reducing PDE 2 activity in PC12 cells by over 50% after 24 h, suggesting a phosphoprotein-mediated regulation of PDE 2 activity. Protein kinase inhibitor effects were difficult to assess due to their direct interaction with the PDE in cell lysates. To study phosphorylation in PDE 2 regulation, PDE 2A2 was epitope-tagged, and stable clonal PC12 cell transfectants were isolated (PC12B cells). When combined with metabolically labeled ^{32}P-phosphoproteins *in vivo* or *in vitro*, phosphoproteins of 108, 90, 64, 43, 33 and 19 kDa coprecipitated with epitope-tagged PDE 2A2 in an NGF sensitive manner. A 23-kDa phosphoprotein containing immunoreactive phosphoserine associated with the complex in an NGF independent manner. Phosphothreonine plus phosphotyrosine immunoreactivity at 23, 24, and 64 kDa as well as the phosphotyrosine immunoreactivity at 108, 90, 64, 43, 33, and 19 kDa required NGF or orthovanadate treatment. These proteins are hypothesized to be part of an NGF-regulated complex controlling PDE 2A2 activity.

Keywords: 3′,5′-cyclic nucleotide phosphodiesterase, cyclic AMP, cyclic GMP, nerve growth factor, PC12 cells, PDE 2, phosphoprotein.

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PC12 cells differentiate into a sympathetic neuronal phenotype with either nerve growth factor (NGF) or cyclic AMP treatment (Greene and Tischler 1976; Gunning *et al*. 1981; Heideman *et al*. 1985). NGF only slightly increases cyclic AMP but greatly potentiates the action of agents that normally raise cyclic AMP in these cells (Baizer and Weiner 1985; Knipper *et al*. 1993). The activation of the cyclic AMP response element binding protein (CREB) through cyclic AMP-dependent protein kinase (PKA) is necessary for differentiation and can occur by either cyclic nucleotide phosphodiesterase (EC 3.1.4.17) inhibition, adenylyl cyclase activation, or the NGF activation of the mitogen-activated protein kinase (MAPK) pathway (Grewal *et al*. 1999). Cyclic AMP stimulates the low molecular weight GTPase Rap1, which along with PKA is required for the long-term stimulation of the MAPK pathway caused by NGF (Yao *et al*. 1998; York *et al*. 1998). The cyclic AMP stimulation of Rap1 uses at least two mechanisms. Cyclic AMP activates the cyclic AMP-binding GTPase exchange factor acting on Rap1 and causes the phosphorylation of Rap1 by PKA (de Rooij *et al*. 1998; Kawasaki *et al*. 1998). The cellular mechanisms by which neurotrophin receptors

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Abbreviations used: BSA, bovine serum albumin; CREB, cyclic AMP response element binding protein; EHNA, erythro-9-(2-hydroxy-3-nonyl)-adenine; IBMX, isobutyl methyl xanthine; MAPK, mitogen-activated protein kinase; NGF, nerve growth factor; ORF, open reading frame; PKA, cyclic AMP-dependent protein kinase; SDS, sodium dodecyl sulfate.

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modulate cyclic AMP are unknown. PKA, in conjunction with other protein kinase pathways also stimulated by NGF, increases the transcription of genes in PC12 and other neuronal cells by the activation of the cyclic AMP response element binding transcription factor, CREB (Riccio *et al*. 1999; Kaplan and Miller 2000).

PDE 2 is a major cyclic GMP-stimulated cyclic nucleotide phosphodiesterase (E.C. 3.1.4.17) of neuronal tissues and the PC12 cell (Sonnenburg *et al*. 1991; Repaske *et al*. 1993; Sadhu *et al*. 1999). PDE 2 hydrolyzes cyclic AMP and cyclic GMP with similar V_{max}, but the enzyme from PC12 cells has a lower K_m for cyclic GMP than cyclic AMP (Whalin *et al*. 1991). When the enzyme is allosterically stimulated by cyclic GMP binding, its apparent K_m for cyclic AMP is reduced by about four-fold. PDE 2 has two cyclic GMP binding sites per dimer with an apparent K_d for cyclic GMP binding of 0.2 μM at the high affinity site (Stroop and Beavo 1991). PDE 2 has extensive structural homology with two other cyclic GMP binding PDEs, PDE 5 (in smooth muscle) and PDE 6 (in photoreceptors) (Charbonneau *et al*. 1990). Unlike related cyclic GMP binding phosphodiesterases, no modulatory proteins copurify from cyclic GMP affinity resins with the particulate, detergent soluble, PDE 2A2 (Whalin *et al*. 1988; Murashima *et al*. 1990). Cyclic GMP stimulates cyclic AMP hydrolysis by PDE 2 by binding to an allosteric noncatalytic cyclic nucleotide binding domain on PDE 2 similar to those on PDEs 5 and 6 (Soderling and Beavo 2000).

A cyclic AMP requirement for the differentiation of PC12 cells in response to neurotrophin raised the possibility that cyclic AMP metabolism might be regulated by NGF. The effects of NGF on cyclic AMP hydrolysis in the presence and absence of cyclic GMP were examined. PDE 2 protein and transcript were measured by western and northern analyses to ensure no loss of PDE 2 protein occured. Since receptor tyrosine kinases like the NGF receptor modulate a variety of protein phosphorylation events, potential phosphoprotein interactions were identified. We demonstrate here for the first time that NGF modulates PDE 2 cyclic nucleotide hydrolysis possibly through regulation of the phosphoproteins interacting with PDE 2. We suggest NGF activates a 'switch' to make neuronal cyclic nucleotide metabolism less cyclic GMP sensitive so both nucleotides stimulate neuronal gene transcription.

Experimental procedures

Culture of PC-12 cells

PC12 cells were cultured with or without treatment with 100 ng NGF/mL as described (Greene and Tischler 1976). Cells were plated on 100-mm plates that had been precoated with 50 μg rat tail collagen and 25 μg poly-D-lysine. After 24 h in this media, cells were serum deprived for 24 h before initiating all experiments. They were serum-deprived in Optimem (Life Technologies, Gaithersburg, MD, USA) for 24 h prior to NGF, isobutyl methyl xanthine (IBMX), or sodium orthovanadate treatment in the same media. Untreated cells were maintained in serum-free medium for at least 24 h and in some cases as long as 144 h with no detectable effect on PDE activity (data not shown). All control cultures responded to incubation with 100 ng NGF/mL for 24 h with at least 60% of the cells producing neurites. When incubated with NGF for sustained periods of time, fresh NGF was added every 48 h. NGF and all cell culture products were obtained from Life Technologies (Gaithersburg, MD, USA) or Alomon Laboratories (Jerusalem, Israel) unless noted otherwise. The bicinchoninic acid assay reagents were obtained from Pierce (Rockford, IL, USA). All other chemicals were obtained from Calbiochem (La Jolla, CA, USA), Fisher Scientific (Pittsburgh, PA, USA), or Sigma (St Louis, MO, USA) unless noted otherwise.

Preparation and analysis of RNA

Total RNA was prepared using Triazol™ reagent (Life Technologies) according to the manufacturer's instructions. Samples were electrophoresed, blotted for northern analysis and hybridized according to methods previously described (Bentley *et al*. 1992). To produce PDE 2 mRNA probes, PC12 cell PDE 2A cDNAs were isolated by the polymerase chain reaction (PCR). PC12 cells were treated for 9 days with 100 ng NGF/mL. Total RNA was extracted and processed for poly-A selection and random hexamer-primed first strand cDNA synthesis as described (Bentley *et al*. 1992). A domain of PDE 2A was amplified using a sense oligonucleotide corresponding exactly to bases # 1181–1200 (GenBank accession #U21101) of rat PDE 2A (Yang *et al*. 1994): 5′-AGGCTCTTCTCCAAGTGGC. The PDE 2A antisense oligonucleotide corresponds to bases # 2088–2106 of rat PDE 2A: 5′-CCTCTGTGGTCCAGGTCG. This oligonucleotide pair is predicted to amplify a PCR product of 925 bases encoding PDE 2A2 residues 383–689. The cDNA produced overlaps codons conserved in both the allosteric cyclic GMP-binding domain and the catalytic domain. The PC12 PDE 2A2 is identical to the described rat brain PDE 2A2 sequence. PCR-derived cDNAs were extracted from low melting point agarose by direct freeze-crush and subcloned into the pAMP-1 (Life Technologies), the pCR 3.1 vectors (Invitrogen, Carlsbad, CA, USA), or the pGEM T vectors (Promega, Madison, WI, USA). Plasmids were sequenced using dideoxy nucleotide terminators with Sequenase, or in automated sequencing reactions using Taq polymerase (Wilson *et al*. 1990; Bentley *et al*. 1992). The cDNAs were used as templates for random priming reactions using [α-^{32}P]dATP and hybridized to total RNA in northern analysis (Bentley *et al*. 1992).

CDNA cloning of a PC12 cell PDE 2A

About 1×10^6 plaques from an undifferentiated PC12 cell λZAPII cDNA library (Boulter and Gardner 1989) were screened with a PCR product (pAMP 2 A:383–689). Twenty-two positives were obtained. The largest was pSK-5.19 A, encoding the open reading frame of rat PDE 2A beginning at base + 810 of the open reading frame (ORF) through the termination codon and 198 bases of 3′ untranslated sequence (see Fig. 1). Another clone used to assemble a full-length contiguous PDE 2A ORF was designated pSK1.22.N (bases + 350 to + 1842). Sequencing throughout these clones verified a single PDE 2 isoform identical to that of rat brain, PDE 2A2.

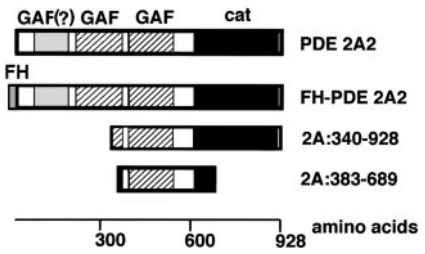

Fig. 1 PDE 2A cDNA isolated from a PC12 library is identical to the rat brain isoform. PDE 2 contains an unique amino terminus distinguishing the PDE 2A1, 2A2, and 2A3 splice variants, tandem conserved cyclic GMP binding or GAF sites (cross hatched domains), and a catalytic domain with residues conserved among PDE gene families 1–7 and 10–11 (domains in black). PDE 2A2 is unique with N-terminal sequences that loosely conform with the GAF consensus sequence. The PCR product initially produced to screen cDNA libraries and for northern analysis (2 A:383–689) encodes part of the second cyclic GMP-binding domain and the catalytic domain. A PC12 cell λZAPII library and PCR overlap mutagenesis were used to clone 2 A:340–928, full-length PDE 2A2, and an N-terminal mutant with a FLAG epitope tag and a polyhistidine marker (FH-PDE 2A2).

Assessment of neurite formation

Using described criteria (Greene *et al*. 1987), neurites were considered cellular processes greater than a cell body diameter long through the geometric center of the cell. Four fields of > 100 adherent cells were inspected for neurites, which were not counted when greater than three cells clustered together. The per cent cells with neuritic processes were averaged to give a single value per plate.

Phosphodiesterase assay

After aspiration of the culture medium, a 100-mm plate was washed once with 10 mL of phosphate-buffered saline (137 mM NaCl, 10 mM Na_2HPO_4, 1 mM KH_2PO_4, 2.7 mM KCl, pH 7.4) at 21°C. The saline was immediately aspirated. Adherent cells were incubated for 30 min at 2–4°C in 20 mM imidazole acetate, 20 mM benzamidine HCl, 20 mM NaF, 2.5 mM EDTA, pH 6.5 and harvested with scraping at 2–4°C. All homogenates were kept at or below this temperature until assay. Some experiments also used 1% polyoxyethylene 9 lauryl ether (Lubrol12A9; Sigma) as a detergent. Cells were disrupted on ice by three bursts of 10 s each from the maximum intensity setting of a Bransom Ultrasonics (Danbury, CT, USA). Protein concentrations were determined by the bicinchoninic acid method (Smith *et al*. 1985). The remainder of the material was adjusted to 25% sucrose and aliquoted for storage at − 70°C until assayed. PDE isoenzyme activities were determined by described methods using 20 mM $MgCl_2$ with no more than 0.5 mM EDTA and [^{3}H]cyclic AMP or [^{3}H]cyclic GMP as the substrate (Sonnenburg *et al*. 1991). All 'unstimulated' and 'cyclic GMP-stimulated' assays were measured in the presence of 2 mM EGTA and no added calcium to avoid PDE 1 stimulation. All 'cyclic GMP-stimulated' assays contain 2 μM added cyclic GMP with [^{3}H]cyclic AMP as the substrate at a final 1 μM total cyclic AMP. All points shown represent product formation linear with protein and time of assay. Statistical significance of sample differences given standard errors of the mean for at least three independent samples performed in triplicate was determined using Student's *t*-test.

NGF deprivation

Cells were plated at a density of 1 X10^5/100 mm dish, and cultured overnight in 10% fetal calf serum, 5% horse serum. They were transferred into 5% horse serum medium incubated again overnight, and treated the next day without or with NGF. After 48 h in NGF, some plates were washed five times with 10 mL of serum-free medium and transferred into 10 mL of 5% horse serum alone as 'NGF deprived'.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and western analysis

SDS–PAGE was performed at high acrylamide : bis-acrylamide ratio (19 : 0.1) for resolution of both high and low molecular weight proteins (Hamilton *et al*. 1993). Samples were made to a final concentration of 1% SDS, 100 mM Tris HCl pH 6.5, 12.5% sucrose, 5% β-mercaptoethanol, and boiled for 5 min. Samples were electrophoresed on polyacrylamide gels (12.5% or 14% acrylamide) and processed for western analysis as described for antibodies raised against PDE 2A1 (Juilfs *et al*. 1997). Antibodies to PDE 1B, PDE 5, phosphoserine, and phosphothreonine were from Chemicon International, Inc. (Temecula, CA, USA). Antiphosphotyrosine was from Transduction Labs (Lexington, KY, USA). Phosphoamino acid antibodies were specific by competition with 100 μM phosphoamino acid (Sigma). Biotinylated anti FLAG antibody M2 was obtained from Sigma. Unlabeled FLAG antibody was obtained from Stratagene (La Jolla, CA, USA). Antirabbit IgG, antibiotin, anti CREB, and antiphosphoserine 133 CREB were obtained from New England Biolabs (Beverly, MA, USA). Immunoglobulin was stripped from westerns for probing with multiple antibodies by incubation with agitation in 0.2 N NaOH, 1% SDS for 10 min at 25°C followed by 0.4 M Tris pH 6.5, 5% β-mercaptoethanol, 1% SDS at 70°C for 30 min. After washes in deionized water, the blot was blocked and redeveloped to ensure no residual antibody was present.

Image analysis and densitometry of autoradiographs and enhanced chemiluminescent images

A Kodak digital camera was used to capture images of autoradiographs, which were analyzed directly with Kodak Digital Science and NIH Image vs. 1.62 software on a Macintosh platform.

Protein kinase and phosphatase inhibitor treatment of PC12 cells

All homogenizations were carried out in 20 mM NaF. Some experiments included treatment of intact cells with sodium orthovanadate for 24 h prior to homogenization, with or without NGF. These agents were added at the same time and washed out with PBS as described above. When protein kinase inhibitors were added with NGF they were introduced 1 h before NGF treatment.

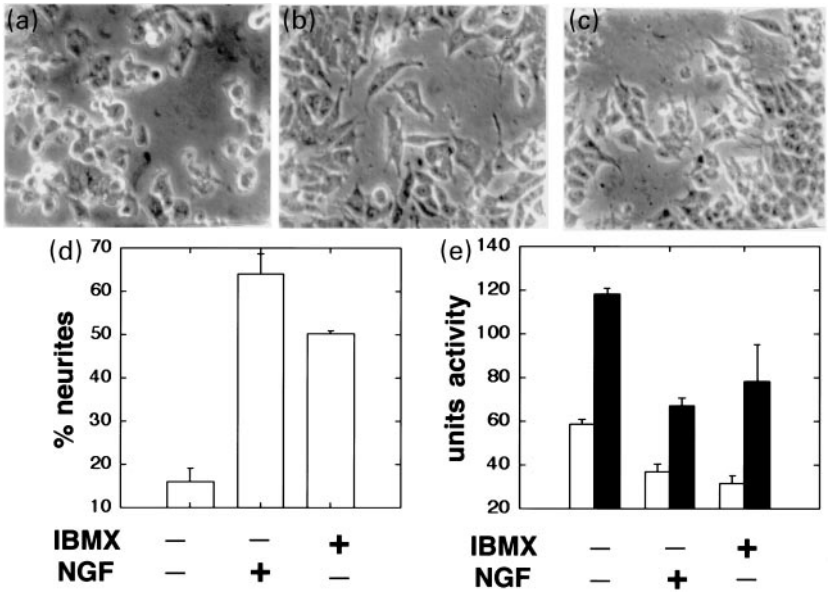

Fig. 2 NGF lowers cyclic GMP-stimulated cyclic AMP hydrolysis under conditions where neurite formation is observed. PC12 cells were grown without (a) or with 100 ng NGF/mL (b) or 0.1 mM isobutyl methyl xanthine (IBMX) for 24 h (c). In (d), the percentage of cells in each treatment with neurites are indicated with the standard error of the mean (SEM) for $N = 3$. In (e), washed cells were harvested in imidazole buffer containing 1% Lubrol12A9, NaF, and protease inhibitors. Total homogenates were assayed with [^{3}H] cyclic AMP for phosphodiesterase activity at 1 μM cyclic AMP in the absence (open bar) or the presence (closed bar) of 2 μM cyclic GMP. A unit is given 1 pmol cyclic AMP hydrolyzed/min/mg protein. SEMs are shown for $N = 3$.

Construction of PDE 2A2 for expression studies and production of a PC12 cell line with a FLAG-epitope (DYKDDDDK) tagged PDE 2A2

The epitope tagged PDE was produced to allow precipitation and affinity purification of active protein by a commercially available antibody. The FLAG epitope and a 10-residue histidine (H10) tag was introduced after the start Met in the following oligonucleotide encoding the first eight codons of PDE 2A2 with an additional 5′ EcoRI site: 5′-GAATTCAGCATGGACTACAAG-GACGACGATGACAAGCATCACCATCACCATCACCATCAC-CATCACGTCCTGGTGTTGCACCACATCC. An identical sense oligonucleotide encoding the same sequence without the EcoRI site or the epitope encoding codons, 5′-AGCATGGTCCTGGTGTTG-CACCACATCC was also made. An antisense oligonucleotide to bases + 717 to + 738, 5′-TGTCTCCTGCTGAAGATATCG, was used with Taq polymerase, PC12 first strand cDNA produced as described (Bentley *et al*. 1992), and the sense oligonucleotides to produce 741 and 795 base cDNAs encoding N-terminal PDE 2A2 (pGEM 2 A:1–246) and FLAG-H10 PDE 2A2 (pGEM FH-2 A:1–246) chimeric PCR products. These were initially cloned into the pGEMT vector for sequencing and were used with the λZAP library clones to construct full-length PDE 2A2 open reading frame cDNAs subcloned into pcDNA 3 (Invitrogen) for expression.

Construction of the wild-type PDE 2A2 or FH-PDE 2A2 involved splicing pGEM 2 A:1–246 or pGEM FH-2 A:1–246 cDNA from a 5′ multiple cloning site SstII through the start site and the epitope tag (for the FH-PDE 2A2) to a BamHI site + 597 in the PDE 2A2 sequence into SstII and BamHI cut, λZAPII excised pSK (2 A:114–616) encoding PDE 2A2 nucleotide sequences from + 340 to + 1850. The resulting clone was sequenced from − 3 to + 1850, pSK(2 A:1–616). The SstII to NdeI fragment from − 3 to + 1684 of PDE 2A2 was cloned into the SstII and NdeI sites of λZAPII excised cDNA (from + 560 to + 2989) designated pSK (186–928). The final clones produced were designated pSK (2A2 : 1–928), or pSK(FH-2A2) for the N-terminal epitope tagged mutant, which were sequenced and identical to PDE 2A2 (outside the epitope tag for FH-2A2). The FH-2A2 cDNA was subcloned into pcDNA3 for the generation of stable PC12 cell transfectants.

Transfection of 1×10^5 PC12 cells in a 100-mm dish was performed with Lipofectamine (Life Technologies) according to manufacturers specifications. Ten microliters of Lipofectamine was mixed with 90 μL of serum-free medium in one tube, 10 μg (10 μL) of pcDNA3 : FH-2A2 was mixed with 90 μL serum-free medium in a second tube, and the two solutions mixed to form micelles for 30 min at room temperature. At the end of 30 min, cells were washed twice with 10 mL serum free medium. Then 800 μL serum-free medium was added to the pcDNA3 : FH-2A2 mixture, which was pipetted on the cells. Cells were incubated for 5 h at 37°C and 5% CO_2. At this time 10 mL of complete medium (with 5% heat inactivated horse serum and 10% fetal calf serum) was added to the cells, which were further incubated 18 h at 37°C. Next, the medium was made 400 μg G418/mL. After one week, changing the media daily, greater than 99.9% of the cells became nonadherent and were removed. The G418 concentration was lowered to 200 μg/mL, for isolation of resistant clones. Over the next 2 weeks, eight distinct colonies formed on the plate. Colonies were isolated using cloning rings, trypsinized off the plate, and replated in 30 mm wells. Established cultures were split into 12 aliquots each and diluted serially on 96-well plates to a dilution where only one of the 12 subclones grew. This clone was isolated for further characterization. The clone giving the highest cyclic GMP PDE activity above untransfected cells (about 2 fold) was chosen for further use (PC12B).

Production of a 60-kDa recombinant PDE 2A fusion protein probe

Using a Hind III site at base + 1018 on the PDE 2A2 open reading frame and a 3′ Not I site on the multiple cloning site of the Bluescript II vector, a cDNA fragment from clone pSK5.19 A encoding from + 1018 through the termination codon was inserted into pET 28b (Calbiochem-Novagen, Madison, WI, USA) to

produce pET PDE 2A: 340–928. This cDNA used the pET vector start site to produce the His_6, T7 epitope-tagged PDE 2A: 340–928 probe.

Interaction with PDE 2A fusion proteins and immunoprecipitation with anti T7 epitope conjugated agarose or direct precipitation with anti FLAG agarose

The T7 2 A:340–928 fusion protein was purified by chromatography on Ni-nitrilotriacetic acid agarose (Qiagen, Chatsworth, CA, USA). The protocols varied from the manufacturer's in that extraction buffers used 0.2 M NaCl, 20 mM Tris HCl, 20 mM imidazole pH 7.5, 20 mM benzamidine HCl, 2 × complete protease cocktail (Roche Molecular Biological, Indianapolis, IN, USA), 1% Triton X-100 and 1% Lubrol12A9. The fusion protein eluted at 100 mM imidazoleHCl pH 7.0. Bacterial recombinant fusion protein required Triton X-100 (0.5% (v/v) or greater) to remain soluble on freeze–thawing.

For precipitations, total cell homogenates were prepared and centrifuged at 10 000 *g* for 30 min at 4°C. The supernatant fluid was removed, precleared by incubation of 100–500 μg protein with 20 μL of a 50% (w/v) suspension of anti T7 epitope conjugated agarose (Calbiochem-Novagen, for T7 precipitation) and protein A agarose (Sigma) for 5 min at 4°C followed by a 5-min centrifugation at 10 000 *g* to obtain the precleared supernatant fluid (Studier *et al*. 1990). Between 1 and 5 μg of T7 2 A: 340–928 was precleared by interaction with 20 μL of a 50% (w/v) suspension of G-50 sepharose. For precipitation of proteins binding T7 2 A:340–928, 100–500 μg of PC12 soluble protein was incubated with 1–5 μg of a purified T7 2 A:340–928 fusion protein prepared by standard protocols (Hall *et al*. 1999). The final reaction mixtures contained 12.5% sucrose, 100 mM NaCl, 0.1% (w/v) bovine serum albumin (BSA), 1% (v/v) Lubrol12A9, 0.5% (v/v) Triton X-100, 20 μL 50% (w/v) suspension anti T7 epitope conjugated agarose, 0.05 M imidazole pH 7.0, 20 mM benzamidine HCl, 10 mM NaF, and 1.25 mM EDTA. Anti FLAG M2 antibody (Stratagene) precipitations were similar but performed with 10 μg antibody and 40 μL protein A agarose or 10 μL M2-agarose. Specific binding to the antibody conjugates occured for 1 h at 4°C on a tumbling mixer. The samples were sedimented at 5000 *g* for 5 min, washed three times with saline supplemented with 1% (v/v) Lubrol12A9, 20 mM imidazole, 20 mM NaF, 20 mM benzamidine, and 2.5 mM EDTA. The pellet was finally processed for SDS–PAGE and autoradiography or western analysis.

FLAG epitopes were visualized with the use of biotinylated anti FLAG M2 monoclonal antibody (Sigma). Use of biotinylated anti FLAG M2 required the use of 5% bovine serum albumin as a blocking agent; all other primary antibodies used 5% non fat dry milk as a blocking agent. Ten micrograms of a biotinylated anti FLAG M2 monoclonal is used with about 100 mm^2 of membrane. Anti FLAG immunoreactivity was visualized using a secondary incubation with either a 1/1000 dilution of streptavidin-conjugated horseradish peroxidase (Amersham-Pharmacia, Piscataway, NJ, USA) or antibiotin (New England Biolabs, Inc., Beverly, MA, USA) and an extended duration chemilumenescent substrate from Pierce.

Radiolabeling of PC12 ATP pools with [^{32}P]

PC12 cells were plated onto collagen and poly-D-lysine as discussed above at a density of 10^5 cells/100 mm plate. After 24 h cells were transferred into phosphate-free RPMI 1640 media (Life Technologies) supplemented with 0.5 mCi $H_3[^{32}P]O_4$. Cells were incubated 24 h before the addition of NGF. After the indicated length of time, cells were homogenized and stored at − 70°C until processed for immunoprecipitation experiments.

Results

PDE 2A2 is a cyclic GMP stimulated cyclic AMP phosphodiesterase activity of PC12 cells

The only known cyclic GMP-stimulated cyclic AMP phosphodiesterase is PDE 2, which is enriched in PC12 cells (Whalin *et al*. 1991; Soderling and Beavo 2000). Since cyclic nucleotides are crucial to the NGF response, probes were developed by RT-PCR for cDNA encoding PDE 2 (Fig. 1). The initial cDNA product was designated 2 A:383–689, since it encoded these amino acids of rat PDE 2A2. A full-length PC12 cell derived PDE 2A2 identical in sequence to the previously described PDE 2A2 from rat brain was assembled (Yang *et al*. 1994). This protein has an estimated molecular weight of 104 792 Da and has the conserved C-terminal PDE catalytic domain from amino acids 641–810 (Soderling and Beavo 2000). Amino terminal to this is a highly conserved (consensus *E*-value 1.2×10^{-37}) cyclic GMP binding GAF domain from amino acids 397–546 (Schultz *et al*. 2000). Amino terminal to this GAF domain is a second highly conserved (*E*-value 4.2×10^{-22}) cyclic GMP binding domain from amino acids (228–375). Of all the PDE 2 splice variants, PDE 2A2 is unique in possessing a third GAF-like domain of loose consensus (*E*-value 0.54) from amino acids 50–189.

NGF decreases PC12 phosphodiesterase activity under conditions where neurites are formed

The phosphodiesterase inhibitor IBMX, like NGF, induces neurite formation with activation of some of the same pathways involved with cellular differentiation (Figs 2a–d) (Grewal *et al*. 1999). Either NGF (100 ng/mL) or IBMX (100 μM) treatment lowered PC12 cell cyclic nucleotide phosphodiesterase activity (Fig. 2e). It was found that NGF-treated cells had lost about half of their cyclic GMP-stimulated phosphodiesterase activity compared to control undifferentiated cells (Fig. 2e).

PDE 2 activity rises on NGF deprivation of PC12 cells

A PDE 2 activity decrease is associated with NGF treatment. A CREB-mediated transcription is an essential part of the NGF response. Therefore, PDE 2 activity might be expected to return to normal under conditions of NGF deprivation where CREB dephosphorylation normally occurs (Riccio *et al*. 1999) if PDE 2 regulation was coupled to CREB dephosphorylation. When cells are treated with NGF for 48 h, washed extensively with serum-free medium, and subsequently NGF deprived for 24 h, the cyclic GMP stimulated cyclic AMP hydrolysis is elevated compared to

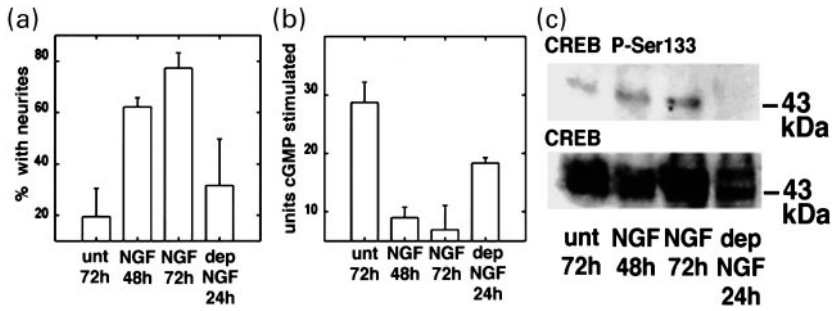

Fig. 3 NGF deprivation increases PDE 2 activity with CREB dephosphorylation and neurite loss. PC12 cells were either untreated (*unt 72 h*) for the 72 h course of the experiment, exposed to NGF for 48 h (NGF 48 h), 72 h (*NGF 72 h*), or exposed to NGF for 48 h and serum deprived further for 24 h (*dep NGF 24 h*). For panel (a), the percentage of cells with neurites under these conditions are assessed and compared to the amount of immunoprecipitable CREB under each of the four conditions. For panel (b), the difference between cyclic GMP stimulated cyclic AMP hydrolysis minus unstimulated cyclic AMP hydrolysis is shown. Panel (c) contains western blot panels showing the amount of immunoreactive CREB and immunoreactive phosphoserine 133 in the same CREB immunoprecipitates.

48 h or 72 h NGF (Fig. 3). Activity does not fully return to the level seen in untreated cells. When CREB is immunoprecipitated from these soluble extracts, processed for western analysis, and probed with an antibody raised against the phosphoserine 133 CREB peptide, 48 and 72 h of NGF treatment are associated with an increase of phosphoCREB immunoreactivity at about 43 kDa. When cells are washed and then NGF- and fetal calf serum deprived for 24 h after 48 h of NGF treatment the amount of immunoreactive phosphoCREB is greatly decreased as is neurite formation. PDE 2 activity reciprocates with CREB phosphorylation and the production of neurites in PC12 cells.

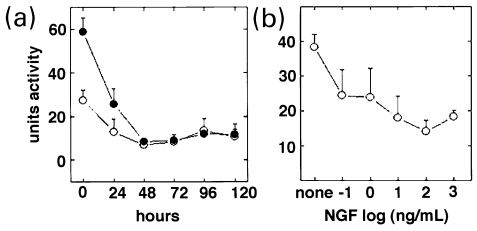

Fig. 4 NGF decreases PDE 2 activity at concentrations associated with neurite production. PC12 cells were either untreated or exposed to 100 ng NGF/mL for 24, 48, 72, 96, or 120 h, washed, homogenized without detergent, and processed for cyclic GMP-stimulated cyclic AMP phosphodiesterase. Shown are the SEM for $N = 6$. In (a), open circles (○) indicate cyclic AMP phosphodiesterase alone; closed circles (●) indicate the hydrolysis of 1 μM [^{3}H]cyclic AMP in the presence of 2 μM cyclic GMP. In (b), PC12 cells were maintained untreated or treated with 0.1, 1, 10, 100, or 1 μg of NGF/mL for 24 h, washed and homogenized and processed for cyclic GMP phosphodiesterase assay. Open circles (○) indicate cyclic GMP phosphodiesterase as indicated by the hydrolysis of 1 μM [^{3}H]cyclic GMP. SEMs are shown for $N = 3$. 1 unit in (a) or (b) is given 1 pmol cyclic NMP hydrolyzed/min/mg protein.

NGF decreases PDE 2 activity at concentrations of NGF producing neurites

The initial loss in activity occurs relatively rapidly (within 24 h) but a slower loss in activity occurs with time (through 120 h). Treatment with 100 ng NGF/mL for 24 h was found to decrease the cyclic GMP-stimulated cyclic AMP PDE activity in PC12 cell homogenates by about 50% (Fig. 4a; $N = 9$ for untreated and 24 h points). Cyclic GMP-stimulated cyclic AMP phosphodiesterase activity values obtained from NGF-treated cells differ at a significance level of $p < 0.001$ when compared to undifferentiated cell activities at times greater than or equal to 48 h of NGF treatment ($N = 6$). With increasing duration of NGF treatment 75–80% of untreated cell cyclic GMP stimulated activity was lost, and after 72 h of NGF the cyclic GMP stimulated activity is not significantly greater than cyclic AMP hydrolysis alone.

We determined whether PDE 2 was the major cyclic GMP-hydrolyzing enzyme of the PC12 cell by examining the sensitivity of the activity to erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA), a PDE 2 selective inhibitor with an IC_{50} of 10 μM (Podzuweit *et al*. 1995). The PDE 1, 5 and 6 selective inhibitor zaprinast was examined for its ability to inhibit PC12 cyclic GMP phosphodiesterase, and zaprinast had an IC_{50} of 18 μM ($N = 4$) (Yan *et al*. 1996). It was found that EHNA most potently ($IC_{50} = 9$ μM) inhibited both cyclic GMP stimulated cyclic AMP and cyclic GMP phosphodiesterase, characteristic of PDE 2A. On fractionation, cyclic GMP stimulated cyclic AMP and cyclic GMP phosphodiesterases eluted from DE-52 at 200 mM NaCl (data not shown). No activity eluting from DE 52 was calcium plus calmodulin stimulated, nor was PDE 1 activity found in whole cell homogenates.

The concentration dependence of NGF on cyclic GMP phosphodiesterase was assessed (Fig. 4b). Concentrations

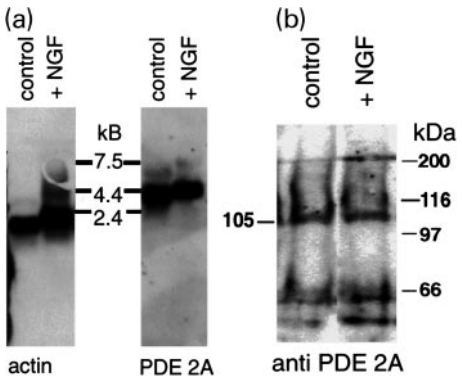

Fig. 5 NGF lowers cyclic GMP-stimulated cyclic AMP hydrolysis without a decrease in PDE 2A mRNA or protein. In (a), RT-PCR derived PC12 cell cDNA corresponding to bases + 1182 to + 2106 of rat PDE 2A2 (encoding amino acids 383–689) was used to produce random primed cDNA probes. Probes were hybridized to 10 μg total RNA collected from untreated cells or cells treated with 100 ng NGF/mL for 24 h. For (b), cells treated without or with NGF for 48 h were processed for western analysis as described in Experimental procedures. Five micrograms of protein was processed using rabbit antibody to bovine adrenal PDE 2A1. Shown is the expected size of PDE 2A2, at 105 kDa.

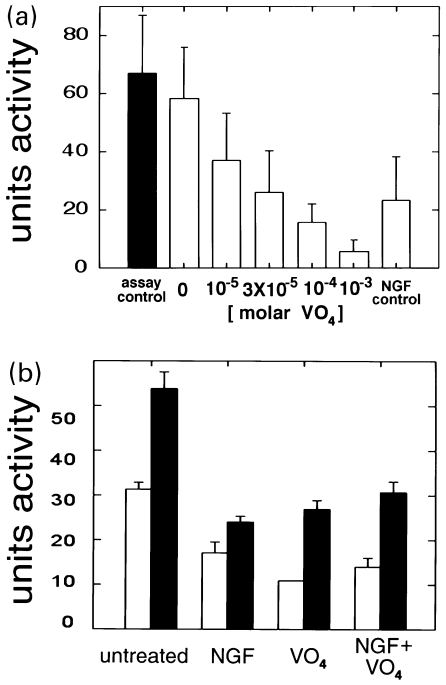

Fig. 6 NGF effects are similar to those of the phosphatase inhibitor sodium orthovanadate, and these effects on PC12 PDE 2 activity are not additive. In (a), either untreated cells (column 1 from the left, *none*), cells treated with increasing amounts of sodium orthovanadate expressed as log [VO_4], or treated with 100 ng NGF/mL (NGF control) for 24 h were washed, homogenized, and processed for cyclic GMP phosphodiesterase activity at 1 μM [^{3}H] cyclic GMP in the presence of detergent as described above. In comparison, extracts of untreated cells assayed with 2 mM Na_3VO_4 directly added to the assay (assay control) are also shown. SEMs are shown for $N = 3$ with units equal to pmol cyclic GMP hydrolyzed/min/mg protein. In (b), after 24 h untreated cells or cells treated with 100 ng NGF/mL (NGF), 30 μM sodium orthovanadate (VO_4), or both (NGF + VO_4), were washed, homogenized, and assayed without detergent for unstimulated (open bar) or cyclic GMP-stimulated (closed bar) cyclic AMP phosphodiesterase at 1 μM [^{3}H] cyclic AMP. Shown are SEM for $N = 4$ with units equal to pmol cyclic AMP hydrolyzed/min/mg protein.

of NGF yielding a half-maximal activity of PC12 cyclic GMP phosphodiesterase were similar to those giving half maximal neurite formation at 24 h, about 10 ng/mL. Only higher concentrations of NGF (10 ng/mL or greater) showed a PDE inactivation of statistical significance ($p < 0.001$).

NGF does not decrease the amount of PDE 2 mRNA or protein in PC12 cells within 48 h

One explanation for loss of PDE 2 activity is that NGF may decrease the amount of cyclic GMP-stimulated PDE 2. To assess PDE 2A mRNA, oligonucleotides corresponding to sequences common to the rat and bovine PDE 2A cDNAs were used to obtain a probe for the PC12 PDE 2 mRNA by RT-PCR (Bentley *et al*. 1992). Upon sequence analysis, this PCR product was found to be an exact match for the rat PDE 2A coding sequence from bases #1181–2106 (Fig. 1). Hybridization was evident to a 4.0–4.4 kB PDE 2A band and weaker bands of higher molecular weight (Fig. 5a). After NGF treatment for 48 h, densitometric analyses indicate the 4 kB PDE 2 mRNA is not diminished significantly (Fig. 5a; densitometry not shown).

The loss of PDE 2 activity is not accompanied by a loss of PDE 2 immunoreactive material (Fig. 4b). Processing 5 μg of a PC12 homogenate for western analysis using a polyclonal monospecific antibody raised to purified bovine PDE 2A1 revealed protein at M_r 105 000, 60 000, and 50 000. This is similar to the major PDE 2A signals observed in rat adrenal or olfactory epithelium with this antibody (Juilfs *et al*. 1997). Lower molecular weight bands vary in different preparations and likely represent PDE 2 proteolysis. PC12 cell PDE 2A immunoreactivity was not decreased by NGF, nor was there any significant decrease in the amount of the peak (on densitometry) or the apparent molecular weight of the anti PDE 2 immunoreactive material with NGF treatment (Fig. 4b). In untreated PC12 mRNA northerns, no signals for PDE 1A, 1B, 1C, or PDE 5 were observed, nor were signals found on western analysis using commercially available antibodies to PDE 1B or PDE 5, nor were calcium-calmodulin stimulated or cyclic GMP-specific activities found on anion exchange

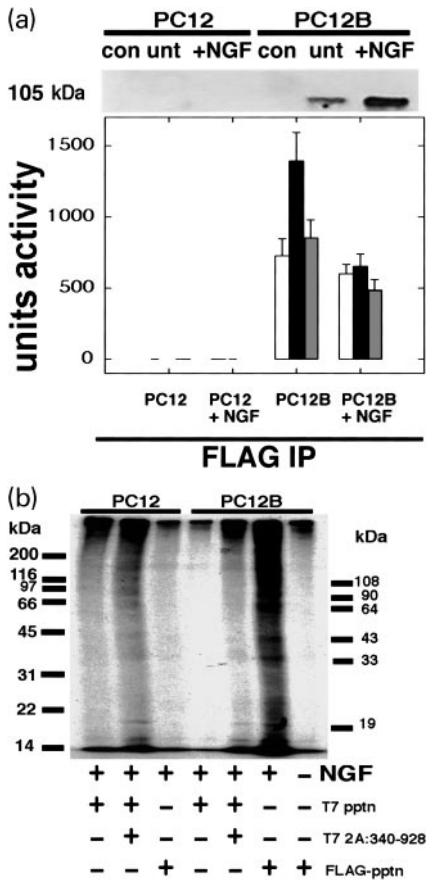

Fig. 7 NGF treatment causes an increase in the ^{32}P content of proteins associating with PDE 2. In (a), PC12 cells or PC12B cells (stable FH-PDE 2A2 transfectants) were serum deprived for 24 h, and the cells were subsequently exposed to 100 ng NGF/mL for 48 h. The detergent-extracted precleared supernatant fluid, 100 μg of cell protein, was incubated with 10 μg of anti FLAG M2, sedimented, washed, and aliquots were processed for SDS–PAGE and western blotting (top panel) or phosphodiesterase activity (bottom panel). A band corresponding to the size of PDE 2A2 at 105 kDa is indicated. For enzyme activity, open bars indicate cyclic AMP hydrolysis, black bars indicate cyclic GMP-stimulated cyclic AMP hydrolysis, and the units shown are pmol cyclic AMP hydrolyzed/min/mL cleared soluble fraction. Grey bars indicate cyclic GMP hydrolysis, and the units shown are pmol cyclic GMP hydrolyzed/min/mL. In (b), ATP pools of PC12 or PC12B cells were equilibrated with 0.25 mCi [^{32}P] orthophosphate under conditions of serum deprivation for 24 h, and the cells were subsequently exposed to 100 ng NGF/mL for 48 h. Cells were lyzed in homogenization buffer with 1% Lubrol 12A9, precleared, and 100 μg of cell protein was interacted with 1 μg of T7 2A2: 340–928 and anti T7 agarose or 10 μg of anti FLAG M2 and protein A agarose, sedimented, washed, and processed for SDS–PAGE and autoradiography.

chromatography from normal or NGF treated cells (data not shown).

In the absence of changes in PDE mRNA or protein levels, a potential mechanism for the loss of PDE 2 activity due to NGF treatment would be a covalent modification of PDE 2 or a regulatory protein. Homogenization using a non-specific phosphatase inhibitor (NaF) was needed to observe NGF inhibition of PDE 2 (data not shown). So, intact cells were treated with a more potent phosphatase inhibitor to reproduce the NGF effect.

Incubation of intact cells, but not cell extracts, with phosphatase inhibitors decreases the activity of PDE 2

Sodium orthovanadate is an effective tyrosine phosphatase inhibitor (Gordon 1991). When PC12 cells are treated for 24 h with 30 μM sodium orthovanadate, cyclic GMP PDE activity decreases to a level equivalent to 24 h incubation with NGF (Fig. 6a). Orthovanadate (up to 1 mM) caused over 90% inhibition of cyclic GMP PDE in adherent cells. Addition of 2 mM orthovanadate to untreated cell homogenates has no effect on PDE 2 activity (Fig. 6a). At 100 ng/mL NGF, 30 μM orthovanadate does not further surpress PDE 2 activity (Fig. 6b). Since orthovanadate lowered PDE 2 only during incubation with intact cells, a regulatory mechanism involving protein phosphorylation was suggested.

Tyrosine kinase and protein kinase C inhibitors have been used to demonstrate protein kinase pathways, but these compounds directly inhibit and activate phosphodiesterases depending on the isozyme and the inhibitor concentration (Eckly Michel *et al*. 1997; Nichols and Morimoto 2000). We found that genistein (100 μM), staurosporine (10 nM), PD98059 (100 μM), SB203580 (10 μM), and calphostin C (100 nM) all had direct interactions with the enzyme (data not shown). PDE-kinase inhibitor interaction dictates a cautious interpretation of kinase regulation of PDE activity *in vivo*.

Under conditions where NGF inhibits PC12 cyclic GMP PDE, phosphate containing proteins are observed to interact with recombinant PDE 2 proteins

To assess PDE 2 phosphoprotein regulation by NGF, a FLAG epitope tagged PDE 2A2 mutant was constructed under the control of a CMV promoter in a G418 resistance vector (FH-PDE 2A2, Fig. 1) and transfected into PC12 cells. One clonal cell line was obtained by limiting dilution of a G418 resistant colony and designated PC12B. This clonal cell line had twice the specific activity of cyclic GMP phosphodiesterase of PC12 cells and 50% of the cyclic GMP phosphodiesterase immunoprecipitated with anti FLAG M2 antibody (data not shown). PDE activity was lowered by 0.1% SDS, so a washing protocol using 1% Lubrol12A9 and 0.1% Tween was developed to preserve PDE 2 activity. Under these conditions, control PC12 cell PDE 2 activity was not precipitated (Fig. 7a) while PC12B PDE 2 activity and immunoreactivity could be observed. NGF treated PC12B cells had less immunoprecipitated cyclic GMP stimulated cyclic AMP PDE activity without loss of FLAG immunoreactivity on western

analysis, or any shift in the apparent molecular weight of the FLAG immunoreactivity with NGF treatment (Fig. 7A). Some interaction of the anti FLAG with material migrating at 60 kDa was evident but unchanged with NGF treatment (data not shown) suggesting the N-terminal FLAG epitope is not lost by proteolysis.

To assess the role of protein phosphorylation in PDE 2 regulation, cellular ATP pools were equilibrated with [^{32}P]-orthophosphate during serum deprivation for 24 h. The cells were exposed to NGF and lyzed to obtain a detergent soluble, protein A agarose and anti T7 epitope agarose precleared fraction. This was precipitated with anti FLAG or incubated with a T7 epitope tagged recombinant PDE 2 fusion protein isolated from *Escherichia coli* (T7 2 A:340–928, Fig. 1) and precipitated with the anti T7 epitope antibody. An increase in phosphoproteins (108, 90, 64, 43, 33 and 19 kDa) associating with added T7 2A2 : 340–928 was evident after 24 h of incubation with NGF in either cell line (Fig. 7b). While anti FLAG M2 precipitated none of these proteins from PC12 controls, the FLAG antibodies precipitated multiple high molecular weight proteins from PC12B cells as well as proteins at 108, 90, 64, 43, 33 and 19 kDa in an NGF-dependent manner (compare lanes 3 and 6; Fig. 7b). The lighter precipitation pattern with the protein added *in vitro* could be the result of better protein interaction with a full-length ORF (the T7-tagged PDE lacks the first 340 amino acids found in FH-PDE 2A2), cotranslational association, or an increased association intracellularly due to a higher effective concentration of the FH PDE 2 in PC12B cells.

Similar interacting phosphoproteins associate with recombinant PDE whether it is directly added to a cell extract or expressed in the cell *in vivo*. The NGF-sensitive increase in phosphoprotein association with PDE 2 may reflect a greater turnover of phosphate on protein after NGF treatment or the net increase in the synthesis of proteins able to bind PDE 2A. To examine whether this change in phosphoprotein association exhibited the same NGF concentration dependence as PDE 2 inactivation, nonradioactive and metabolically labeled PC12B cells were treated with a range of NGF

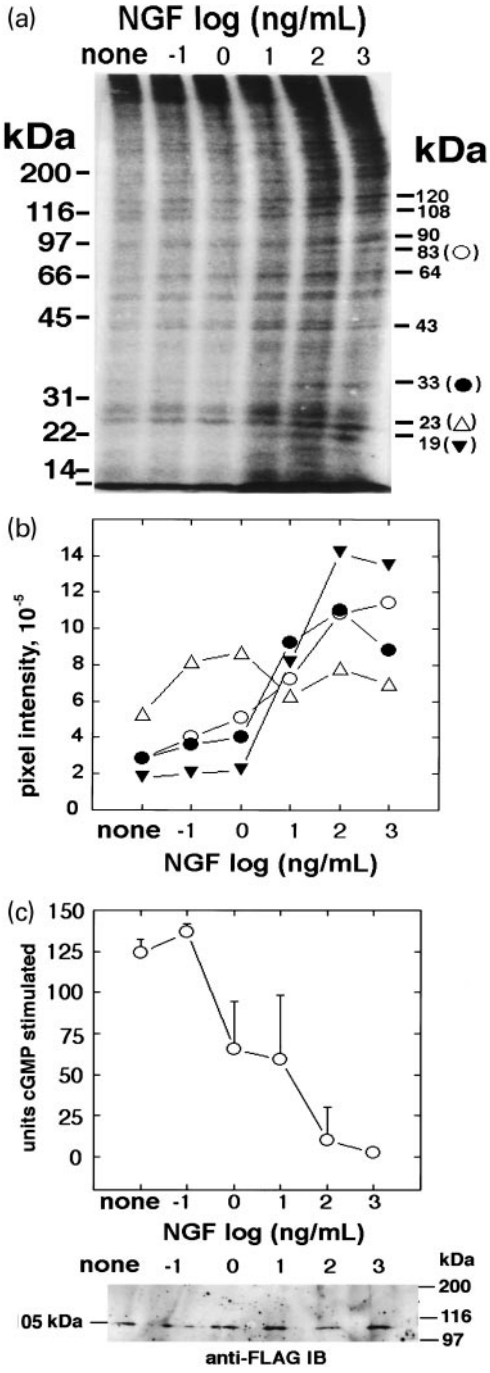

Fig. 8 NGF causes a differential increase in phosphoprotein association with PDE 2A where it causes a decrease in cyclic GMP hydrolysis. In (a), PC12B cells were preloaded with 1 mCi [^{32}P]-orthophosphate for 24 h with serum deprivation and for 48 h with the indicated concentration of NGF. A detergent extract was precleared with protein A agarose, anti T7 agarose, and precipitated with anti FLAG M2-protein A agarose complexes. The pellet washed as and processed for SDS–PAGE and autoradiography. Bands selected for further study by image analysis are indicated by symbols. In (b), the autoradiograph bands shown in (a) at M_r = 85 000 (○), 35 000 (●), 23 000 (Δ), and 19 000 (▼;) are photographed in a digital camera and analyzed for pixel intensity changes in response to increasing NGF. In (c), cells were treated identically to those described in (a) except [^{32}P]-*ortho*-phosphate was omitted. After being incubated with the indicated amount of NGF for 48 h and processed for FLAG immunoprecipitation, aliquots of the precipitate were processed for anti FLAG western blots (bottom panel) while other aliquots were assayed for cyclic AMP phosphodiesterase using [^{3}H] cyclic AMP at 1 μM with 2 μM cyclic GMP (top panel). Units shown are pmol cyclic AMP hydrolyzed/min/mL extract assayed.

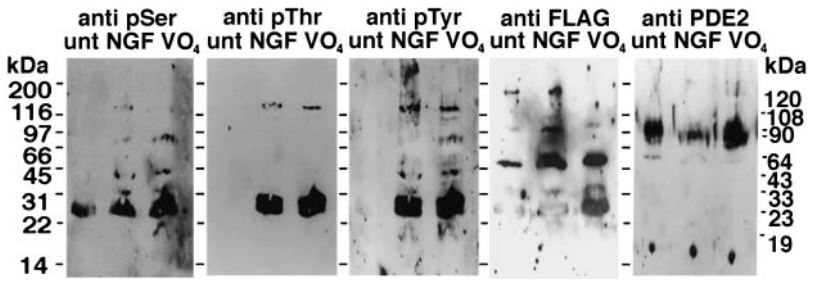

Fig. 9 NGF or sodium orthophosphate cause differential increases in phosphoserine, phosphothreonine, or phosphotyrosine immunoreactivity of FH-PDE 2A2 associated proteins. Soluble fractions (1 mg protein) from either untreated cell (*con*), cells treated for 24 h with 100 ng NGF/mL (*NGF*), or 30 μM sodium orthovanadate (*VO_4*) were precleared with anti T7 agarose plus protein A agarose, precipitated with anti FLAG M2 agarose, and processed for western analyses. The blot was probed sequentially with antiphosphoserine (pSer), antiphosphothreonine (pThr), antiphosphotyrosine (pTyr), anti FLAG M2 (anti FLAG) and anti PDE 2 (PDE 2) and exposed from 1 to 10 min depending on the antibody. Between different antibodies the blot was stripped of IgG as described in the Experimental procedures section, washed, and developed again with chemilumenescent peroxidase substrate to ensure none of the previous conjugate probe remained.

concentrations, detergent extracted, and processed for FLAG immunoprecipitation.

NGF causes an increase in PDE 2 associated phosphoproteins at concentrations producing a loss of PDE 2 activity

PC12B cells incubated with concentrations of NGF which lower PC12 PDE 2 activity had an increased amount of ^{32}P-containing proteins at 108, 90, 64, 43, 33 and 19 kDa (and others) interacting with FH-PDE 2 expressed *in vivo* (Fig. 8a). The 108 kDa protein might represent FH-PDE 2A2, but it is labeled to some extent even in untreated cells (Fig. 8a). FH-2A2 purification on Ni-NTA agarose followed by cyclic GMP sepharose adsorption and elution revealed no radiolabeled bands (data not shown). Digital quantitation of protein interactions showed that several phosphoproteins were immunoprecipitated with PDE 2. Some phosphoproteins were sensitive to NGF treatment, such as the proteins at $M_r = 84\,000$, 33 000, and 19 000 (Fig. 8b), while others were insensitive to NGF treatment such as the $M_r = 23\,000$ protein. Half maximal effects of NGF, for the NGF sensitive phosphoproteins, occured at approximately 10 ng NGF/mL. This same concentration of NGF produced half maximal inhibition of anti FLAG precipitable FH-PDE 2 activity (Fig. 8c).

The NGF receptor does not interact with immunoprecipitable FH-PDE 2 (data not shown), but many of anti FLAG: FH-PDE 2 precipitable bands contained phosphotyrosine as evidenced by antiphosphotyrosine (pTyr) interaction (Fig. 9). The differential incorporation of phosphate is strikingly evident when the same blot is sequentially probed, developed, completely stripped of the inital antibodies, and reprobed with another specific antibody as in Fig. 9. NGF or orthovanadate treatment increase the intensity anti pTyr staining of bands at 19, 24, 33, 43, 64, 83, and 90 kDa. Some of these proteins, like the 23 kDa band, also interact with antiphosphoserine (pSer) without changing on NGF treatment. The antiphosphothreonine (pThr) and antiphosphotyrosine (pTyr) staining proteins at 24 and 120 kDa are very sensitive to NGF or orthovanadate treatment of the whole cell. Many of the phosphoproteins visible on *in vivo* labeling are poorly resolved on immunostaining, indicating these bands may be low abundance proteins. Like PDE 2, the FLAG-tagged PDE 2A2 protein is partially proteolyzed. The anti FLAG interacts with the some of the same sized proteolytic products (around 60–65 kDa) as the polyclonal PDE 2 antibody, but their relative intensities vary due to their target epitopes. The monoclonal raised to the N-terminal FLAG epitope and the polyclonal rabbit antibody raised to the whole PDE 2A1 protein would be expected to interact differentially. These results potentially explain why the FLAG epitope worked so much better in our hands to precipitate an NGF-sensitive PDE : the FLAG epitope provides a 'handle' for the antibody to recognize that NGF-regulated proteins do not compete for in binding to the phosphodiesterase.

Discussion

NGF treatment of PC12 cells has effects on cyclic nucleotide metabolism, and PDE 2 is a major cyclic AMP phosphodiesterase in PC12 cells (Whalin *et al*. 1991). Some effects may be mediated by regulation of PDE 2, since NGF reduces PDE 2 within 24 h by about 50% and to about 80% on sustained NGF treatment. NGF does not reduce either PDE 2 mRNA or protein. NGF treatment correlates with an increased 32-phosphorous content of specific proteins that copurify with the enzyme through immunoprecipitation. Proteins of the same size interact directly *in vitro* with added recombinant PDE 2 as well as *in vivo* with the epitope tagged full-length PDE 2A2, although *in vivo* expressed

full-length PDE 2 binds PC12 phosphoproteins better than PDE 2: 340–928 added *in vitro*. Whether this is due to additional sequence of the *in vivo* expressed protein or cotranslation is unclear at this time.

The PC12 proteins associated with PDE 2 are not likely to be known subunits of other PDE isoforms. Detergent solubilized, anion exchange purified PDE 2 from brain does not elute from cyclic GMP affinity resins with detectable levels of bound proteins (Murashima *et al*. 1990). We found similar results using anion exchange purified PDE 2 extracts from detergent solubilized PC12 cells (data not shown).

Other PDEs form protein complexes modulating their activity and localization through protein kinase pathways. PDE 4 splice variants have regulatory interactions localized in their unique amino termini. PDE 4D4 and PDE 4A5 bind to src, lyn, and fyn kinase SH3 domains to alter the conformation of the PDE catalytic domain as detected by increased rolipram potency (Houslay and Kolch 2000).

While a PKA phosphorylation has been described for PDE 2A2 and not PDE 2A1, this phosphorylation has no effect on enzymatic activity (Whalin *et al*. 1988). Three splice variants of a single PDE 2 gene are known (Rosman *et al*. 1997). PDE 2A1, 2, and 3 are nearly identical except for their 25, 37, and 44 residue amino termini. The PDE 2A2 gene product, found largely in the brain, partitions into particulate and soluble fractions (Whalin *et al*. 1988; Murashima *et al*. 1990; Yang *et al*. 1994).

NGF has more complex effects on cyclic nucleotide metabolism than simply on PDE 2. PDE 4 is also a major PC12 PDE isoenzyme (Chang *et al*. 1997). NGF-mediated activation of ERKs would be expected to inhibit some PDE 4 isoforms (Houslay and Kolch 2000).

NGF decreases soluble guanylyl cyclase activity. Cyclase activity and protein are lowered within 24 h after a rapid (2–4 h) decrease in soluble guanylyl cyclase $\alpha 1$ and $\beta 1$ mRNA expression (Liu *et al*. 1997). NO acts like millimolar cyclic GMP to increase the efficacy of NGF, but also stimulates PC12 neurite formation independently of cyclic GMP (Hindley *et al*. 1997; Phung *et al*. 1999). NO donors do not lower PDE 2 or change the ability of NGF to lower PDE 2 activity (data not shown).

The physiological need for decreasing PDE activity in response to NGF may be to increase the efficacy of agents which activate adenylyl and guanylyl cyclase, supporting the activation of other cyclic nucleotide mediated processes, such as CREB phosphorylation. NGF is hypothesized to activate a 'switch' to make neuronal cyclic nucleotide metabolism less cyclic GMP sensitive. NGF 'primes' the cell for cyclic AMP regulation through PDE 2 inhibition. We suggest that the regulation of PDE 2 activity occurs through association of phosphoproteins with PDE 2 in response to NGF. The pathway by which phosphorylation and association of these proteins alters phosphodiesterase activity in PC12 cells should reveal novel PDE regulatory mechanisms affecting cyclic nucleotide metabolism.

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References

Baizer L. and Weiner N. (1985) Nerve growth factor treatment enhances nicotine-stimulated dopamine release and increases in cyclic adenosine 3′ : 5′-monophosphate levels in PC12 cell cultures. *J. Neurosci.* **5**, 1176–1179.

Bentley J. K., Kadlecek A., Sherbert C. H., Seger D., Sonnenburg W. K., Charbonneau H., Novack J. P. and Beavo J. A. (1992) Molecular cloning of cDNA encoding a '63'-kDa calmodulin-stimulated phosphodiesterase from bovine brain. *J. Biol. Chem.* **267**, 18676–18682.

Boulter J. and Gardner P. D. (1989) Practical approaches to molecular cloning of nicotinic acetylcholine receptor genes. In: *Methods in Neurosciences*, **Vol. 1**, Genetic Probes (Conn P. M., ed.), pp. 328–363, Academic Press, Orlando, FL.

Chang Y. H., Conti M., Lee Y. C., Lai H. L., Ching Y. H. and Chern Y. (1997) Activation of phosphodiesterase IV during desensitization of the A2A adenosine receptor-mediated cyclic AMP response in rat pheochromocytoma (PC12) cells. *J. Neurochem.* **69**, 1300–1309.

Charbonneau H., Prusti R. K., LeTrong H., Sonnenburg W. K., Mullaney P. J., Walsh K. A. and Beavo J. A. (1990) Identification of a noncatalytic cGMP-binding domain conserved in both the cGMP-stimulated and photoreceptor cyclic nucleotide phosphodiesterases. *Proc. Natl Acad. Sci. USA* **87**, 288–292.

Eckly-Michel A. E., Le Bec A. and Lugnier C. (1997) Chelerythrine, a protein kinase C inhibitor, interacts with cyclic nucleotide phosphodiesterases. *Eur. J. Pharmacol.* **324**, 85–88.

Gordon J. A. (1991) Use of vanadate as protein-phosphotyrosine phosphatase inhibitor. *Methods Enzymol.* **201**, 477–482.

Greene L. A. and Tischler A. S. (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl Acad. Sci. USA* **73**, 2424–2428.

Greene L. A., Aletta J. M., Rechenstein A. and Green S. H. (1987) PC12 pheochromocytoma cells: culture, nerve growth factor treatment, and experimental exploitation. *Methods Enzymol.* **147B**, 207–216.

Grewal S. G., York R. D. and Stork P. J. S. (1999) Extracellular-signal-regulated kinase signaling in neurons. *Curr. Opin. Neurobiol.* **9**, 544–553.

Gunning P. W., Landreth G. E., Bothwell M. A. and Shooter E. M. (1981) Differential and synergistic actions of nerve growth factor and cyclic AMP in PC12 cells. *J. Cell. Biol.* **89**, 240–245.

Hall K. U., Collins S. P., Gamm D. M., Massa E., DePaoli-Roach A. A. and Uhler M. D. (1999) Phosphorylation-dependent inhibition of protein phosphatase-1 by G-substrate. *J. Biol. Chem.* **274**, 3485–3495.

Hamilton S. E., Prusti R. K., Bentley J. K., Beavo J. A. and Hurley J. B.

(1993) Affinities of bovine photoreceptor cGMP phosphodiesterases for rod and cone inhibitory subunits. *FEBS Lett.* **318**, 157–161.

Heidemann S. R., Joshi H. C., Schechter A., Fletcher J. R. and Bothwell M. (1985) Synergistic effects of cyclic AMP and nerve growth factor on neurite outgrowth and microtubule stability of PC12 cells. *J. Cell. Biol.* **100**, 916–927.

Hindley S., Juurlink B. H. J., Gysbers J. W., Middlemiss P. J., Herman M. A. R. and Rathbone M. P. (1997) Nitric oxide donors enhance neurotrophin-induced outgrowth through a cGMP-dependent mechanism. *J. Neurosci. Res.* **47**, 427–439.

Houslay M. D. and Kolch W. (2000) Cell-type specific integration of cross-talk between extracellular signal-regulated kinase and cAMP signaling. *Mol. Pharmacol.* **58**, 659–668.

Juilfs D. M., Fulle H. J., Zhao A. Z., Houslay M. D., Garbers D. L. and Beavo J. A. (1997) A subset of olfactory neurons that selectively express cGMP-stimulated phosphodiesterase (PDE2) and guanylyl cyclase-D define a unique olfactory signal transduction pathway. *Proc. Natl Acad. Sci. USA* **94**, 3388–3395.

Kaplan D. R. and Miller F. D. (2000) Neurotrophin signal transduction in the nervous system. *Curr. Opin. Neurobiol.* **10**, 381–391.

Kawasaki H., Springett G. M., Mochizuki N., Toki S., Nakaya M., Matsuda M., Housman D. E. and Graybiel A. M. (1998) A family of cAMP-binding proteins that directly activate Rap1. *Science* **282**, 2275–2279.

Knipper M., Beck A., Rylett J. and Breer H. (1993) Neurotrophin induced cAMP and IP3 responses in PC12 cells. Different pathways. *FEBS Lett.* **324**, 147–152.

Liu H., Force T. and Bloch K. D. (1997) Nerve growth factor decreases soluble guanylate cyclase in rat pheochromocytoma PC12 cells. *J. Biol. Chem.* **272**, 6038–6043.

Murashima S., Tanaka T., Hockman S. and Manganiello V. (1990) Characterization of particulate cyclic nucleotide phosphodiesterases from bovine brain: purification of a distinct cGMP-stimulated isoenzyme. *Biochemistry* **29**, 5285–5292.

Nichols M. R. and Morimoto B. H. (2000) Differential inhibition of multiple cAMP phosphodiesterase isozymes by isoflavones and tyrphostins. *Mol. Pharmacol.* **57**, 738–745.

Phung Y. T., Bekker J. M., Hallmark O. G. and Black S. M. (1999) Both neuronal NO synthase and nitric oxide are required for PC12 cell differentiation: a cGMP independent pathway. *Mol. Brain Res.* **64**, 165–178.

Podzuweit T., Nennstiel P. and Muller A. (1995) Isozyme selective inhibition of cGMP-stimulated cyclic nucleotide phosphodiesterases by erythro-9-(2-hydroxy-3-nonyl) adenine. *Cell. Signal.* **7**, 733–738.

Repaske D. R., Corbin J. G., Conti M. and Goy M. F. (1993) A cyclic GMP stimulated cyclic nucleotide phosphodiesterase gene is highly expressed in the limbic system of rat brain. *Neuroscience* **56**, 673–686.

Riccio A., Ahn S., Davenport C. M., Blendy J. A. and Ginty D. D. (1999) Mediation by a CREB family transcription factor of NGF-dependent survival of sympathetic neurons. *Science* **286**, 2358–2361.

de Rooij J., Zwartkruis F. J., Verheijen M. H., Cool R. H., Nijman S. M., Wittinghofer A. and Bos J. L. (1998) Epac is a Rap1 guanine nucleotide-exchange factor directly activated by cyclic AMP. *Nature* **396**, 474–477.

Rosman G. J., Martins T. J., Sonnenburg W. K., Beavo J. A., Ferguson K. and Loughney K. (1997) Isolation and characterization of human cDNAs encoding a cGMP-stimulated 3′,5′-cyclic nucleotide phosphodiesterase. *Gene* **191**, 89–95.

Sadhu K., Hensley K., Florio V. A. and Wolda S. (1999) Differential expression of the cyclic GMP- stimulated phosphodiesterase PDE 2A in human venous and capillary endothelial cells. *J. Histochem. Cytochem.* **47**, 895–905.

Schultz J., Copley R. R., Doerks T., Ponting C. P. and Bork P. (2000) SMART: a Web-based tool for the study of genetically mobile domains. *Nucleic Acids Res.* **28**, 231–234.

Smith P. K., Krohn R. I., Hermanson G. T., Mallia A. K., Gartner F. H., Provenzano M. D., Fujimoto E. K., Goeke N. M., Olson B. J. and Klenk D. C. (1985) Measurement of protein using bichinchoninic acid. *Anal. Biochem.* **175**, 231–237.

Soderling S. H. and Beavo J. A. (2000) Regulation of cAMP and cGMP signaling: new phosphodiesterases and new functions. *Curr. Opin. Cell Biol.* **12**, 174–179.

Sonnenburg W. K., Mullaney P. J. and Beavo J. A. (1991) Molecular cloning of a cyclic GMP-stimulated cyclic nucleotide phosphodiesterase cDNA. Identification and distribution of isozyme variants. *J. Biol. Chem.* **266**, 17655–17661.

Stroop S. D. and Beavo J. A. (1991) Structure and function studies of the cGMP-stimulated phosphodiesterase. *J. Biol. Chem.* **266**, 23802–23809.

Studier F. W., Rosenberg A. H., Dunn J. J. and Dubendorff J. W. (1990) Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* **185**, 60–89.

Whalin M. E., Strada S. J. and Thompson W. J. (1988) Purification and partial characterization of membrane-associated type II (cGMP-activatable) cyclic nucleotide phosphodiesterase from rabbit brain. *Biochim. Biophys. Acta* **972**, 79–94.

Whalin M. E., Scammell J. G., Strada S. J. and Thompson W. J. (1991) Phosphodiesterase II, the cGMP-activatable cyclic nucleotide phosphodiesterase, regulates cyclic AMP metabolism in PC12 cells. *Mol. Pharmacol.* **39**, 711–717.

Wilson R. K., Chen C. and Hood L. (1990) Optimization of asymmetric polymerase chain reaction for rapid fluorescent DNA sequencing. *Biotechnology* **8**, 184–189.

Yan C., Zhao A. Z., Bentley J. K. and Beavo J. A. (1996) The calmodulin-dependent phosphodiesterase gene PDE 1C encodes several functionally different splice variants in a tissue specific manner. *J. Biol. Chem.* **271**, 25699–25706.

Yang Q., Paskind M., Bolger G., Thompson W. J., Repaske D. R., Cutler L. S. and Epstein P. M. (1994) A novel cyclic GMP stimulated phosphodiesterase from rat brain. *Biochem. Biophys. Res. Commun.* **205**, 1850–1858.

Yao H., York R. D., Misra-Press. A., Carr D. W. and Stork P. J. S. (1998) The cyclic adenosine monophosphate-dependent protein kinase (PKA) is required for sustained activation of mitogen-activated kinases and gene expression by nerve growth factor. *J. Biol. Chem.* **273**, 8240–8247.

York R. D., Yao H., Dillon T., Ellig C. L., Eckert S. P., McClesky E. W. and Stork P. J. S. (1998) Rap1 mediates sustained MAP kinase activation induced by nerve growth factor. *Nature* **392**, 622–626.