

Differential Inhibition of Nerve Growth Factor Responses by Purine Analogues: Correlation with Inhibition of a Nerve Growth Factor-activated Protein Kinase

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Abstract. Purine analogues were used in this study to dissect specific steps in the mechanism of action of nerve growth factor (NGF). Protein kinase N (PKN) is an NGF-activated serine protein kinase that is active in the presence of Mn^{++} . The activity of PKN was inhibited in vitro by purine analogues, the most effective of which was 6-thioguanine (apparent $K_i = 6 \mu M$). Several different criteria indicated that 6-thioguanine is not a general inhibitor of protein kinases and that it is relatively specific for PKN. For instance, it did not affect protein kinases A or C and was without effect on the overall level and pattern of protein phosphorylation by either intact or broken PC12 cells. Since purine analogues rapidly and effectively enter cells, they were also assessed for their actions on both transcription-dependent and -independent responses of PC12

cells to NGF. NGF-promoted neurite regeneration was reversibly suppressed by the analogues and at concentrations very similar to those that inhibit PKN. Comparable concentrations of the analogues also blocked NGF-stimulated induction of ornithine decarboxylase activity. In contrast to its inhibition of neurite regeneration and ornithine decarboxylase induction, 6-thioguanine did not suppress NGF-dependent induction of *c-fos* mRNA expression. Thus, purine analogues such as 6-thioguanine appear capable of differentially suppressing some, but not other actions of NGF. These findings suggest the presence of multiple pathways in the NGF mechanism and that these can be dissected with purine analogues. Moreover, these data are compatible with a role for protein kinase N in certain of these pathways.

SINCE the characterization of nerve growth factor (NGF) (27) as a neurotrophic agent necessary for the development and function of certain peripheral and CNS neurons (11, 21, 26, 32), many events in its mode of action have been revealed (cf. reference 25 for review), but we are still far from a complete understanding of the entire process. The biological effects of NGF comprise both transcription-dependent (15, 24) and -independent responses (4, 18). To provide neuronal differentiation and trophic support, these responses must be coordinated in the cell by means that are still unknown. In this regard, many studies have been performed with the PC12 rat pheochromocytoma cell line (13), a model system that has been extensively used to examine the NGF mechanism of action.

A parameter that is likely to be causally involved in many of the events controlled by NGF in the target cell is the regulation of protein phosphorylation. It is well-documented that NGF, like many other growth factors, promotes rapid changes in the phosphorylation of specific cellular proteins

(1, 18, 19, 34), as well as regulation of several different protein kinase activities (2, 8, 19, 23, 25, 33, 35).

In the present work we have further investigated the possible functional role of a previously described (2, 35, 36, 39). NGF-regulated serine protein kinase, designated protein kinase N (PKN). This enzyme is rapidly activated by NGF in PC12 cells (2, 35) as well as in several nonneural cell lines that possess functional NGF receptors, but that, unlike PC12 cells, lack morphological and a variety of additional responses to the factor (39). In vitro, PKN uses histone Hf1, tyrosine mono-oxygenase, and ribosomal S6 protein as substrates (2, 35), is soluble, is not inhibited by Mn^{++} , and does not require Ca^{++} , cAMP or other cofactors. As demonstrated by a variety of criteria, PKN is distinct from other well-characterized protein kinases (2, 35, 36). Partial isolation of the enzyme has been achieved by FPLC ion-exchange and gel exclusion chromatography (36) and rapid methods for the partial purification and cell-free assay of this kinase from multiple samples have been developed (39).

One potential approach to assessing the functional role of PKN is to use agents that selectively inhibit its activity. We report here that purine analogues can effectively block PKN activity. Moreover, these compounds differentially suppress

1. *Abbreviations used in this paper:* 2-AP, 2-aminopurine; NGF, nerve growth factor; ODC, ornithine decarboxylase; PKA, cyclic AMP-dependent protein kinase; PKC, Ca^{++} /phospholipid-dependent protein kinase; PKN, protein kinase N; 6-TG, 6-thioguanine.

some, but not other, transcription-dependent and -independent responses of PC12 cells to NGF and do so with potencies similar to those with which they inhibit PKN. Such findings both suggest a potential role for PKN in the NGF mechanism of action and indicate the existence of multiple, separable pathways in this mechanism.

Materials and Methods

Materials

Purine analogues were purchased from Sigma Chemical Co. (St. Louis, MO). Stock solutions (1–100 mM) were prepared in RPMI 1640 medium or in water (both adjusted to pH 7.4). In some cases, solubility was achieved by heating the compounds in a boiling waterbath. Histone HFI (lysine rich) was purchased from Organon Teknika-Cappel (Malvern, PA).

Cell Culture

PC12 cells were cultured as previously described on collagen-coated culture dishes in RPMI 1640 medium supplemented with 10% heat inactivated horse serum and 5% FBS (13). Where specified, mouse submaxillary NGF (31) was directly added to cultures from a stock of 250 $\mu\text{g}/\text{ml}$. In some experiments, various purine analogues were added directly to the medium from concentrated stocks prepared in RPMI 1640 medium.

Partial Purification and Assay of PKN

This was carried out as previously described (35, 39). Briefly, cultures (100-mm dishes) were washed twice with a Hepes-buffered modified Krebs-Ringer solution (pH 7.4) and the cells were scraped in 300 μl of ice-cold harvest buffer (50 mM Hepes, pH 7.4, 2 mM EGTA, 10 mM NaF, 10 mM MnCl_2 , 2 mM PMSF, 100 KIU/ml Trasylol), sonicated, and centrifuged at 4°C for 15 min at 60,000 rpm in an ultracentrifuge (model TL-100; SN-941 rotor; Beckman Instruments, Inc., Palo Alto, CA). Soluble proteins were loaded onto Mono S cation exchange resin mini-columns (500 μl of resin packed in 1 ml disposable syringe) mounted on a Super Separator-24 vacuum filtration system (Amersham Corp., Arlington Heights, IL) and activity was eluted as follows. Flow-through fractions were eluted, the columns were each washed with 5 ml of buffer A (extraction buffer, without Trasylol and PMSF), and then with 2 ml of 50 mM NaCl in buffer A; the N-kinase activity was eluted with 2 ml of 300 mM NaCl in buffer A. This step eliminates ~80–90% of total protein and ~50% of histone-phosphorylating activity in the cell extract, with high recovery of PKN activity. Aliquots of the PKN-enriched fraction (40 μl , 1–4 μg of protein) were mixed with 10 μl of a solution containing 5–10 μg of histone HFI and 10 mM MnCl_2 . The phosphorylation assay was started by the addition of 15 μl of a solution containing 1 μCi of [γ - ^{32}P] ATP (3,000 Ci/mmol; New England Nuclear, Boston, MA) and 50 μM ATP. This mixture was incubated for 10 min at 37°C and the reaction was stopped by the addition of 30 μl of 4 \times concentrated electrophoretic sample buffer (35). Samples were incubated for 5 min at 95°C and then subjected to nitrocellulose slot-filtration to separate the phosphorylated histone from free [γ - ^{32}P] ATP (39). Single slots were excised and assessed for radioactivity by liquid scintillation counting. Blanks were determined from samples incubated in parallel without the substrate (histone) or without the PKN fraction. Purine analogues were directly added to the kinase assay from concentrated stocks. Experimental values are expressed as “PKN activity/dish”, i.e., total radioactivity (cpm) incorporated into the histone (less blanks) by the entire PKN-enriched fraction, obtained from one cell culture dish.

Assay of cAMP-dependent Protein Kinase (PKA) and Ca^{++} /Phospholipid-dependent Protein Kinase (PKC)

PKA. Cell extracts were prepared in buffer B (15 mM Na phosphate buffer, pH 6.5, 25 mM Mg acetate, pH 6.5, 15 mM NaF, 2 mM PMSF, 100 KIU/ml Trasylol) as described above for PKN. Aliquots of the extracts (10–30 μg of protein) were mixed with 20 μg /assay of histone III S in 65 μl final volume of buffer A \pm 10 μM cAMP (7). 1 μCi of [γ - ^{32}P]ATP (3,000Ci/mmol; New England Nuclear) in ATP (50 μM final concentration) was added and the phosphorylating reaction was performed at 37°C for 10 min. Samples were then resolved by SDS-PAGE (6–12% linear acrylamide gradients) and processed for autoradiography.

PKC. This was performed as for PKA with the exception that cell extraction was in buffer C (25 mM Tris-Cl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 6 mM Mg acetate, 2 mM PMSF, 100 KIU/ml Trasylol) and the phosphorylating reaction was performed in buffer C \pm 1.5 mM CaCl_2 and with 5 $\mu\text{g}/\text{ml}$ phosphatidylserine and 0.5 $\mu\text{g}/\text{ml}$ diolein (19).

Phosphorylation Studies

These were performed as previously described (16). Briefly, cells (in 35-mm dishes) were incubated with or without NGF in Hepes-buffered modified Krebs-Ringer solution (pH 7.4), in the presence of 100 $\mu\text{Ci}/\text{ml}$ of [^{32}P]orthophosphate for 2–4 h. When indicated, the cultures were preincubated for 1 h with 6-thioguanine (100 μM and 500 μM) or adenine (5 mM) and then subjected to phosphorylation in the continued presence of these drugs. Cells were scraped off the dishes in 200 μl of sample buffer and aliquots (containing equal amounts of TCA-precipitable radioactivity) were subjected to SDS-PAGE on 6–12% acrylamide gradient gels (30 cm long).

Ornithine Decarboxylase (ODC) Activity

This was measured by methods slightly modified from those previously described (14). PC12 cells were cultured overnight on 35-mm dishes in RPMI containing 1% horse serum. NGF was then added for 6 h at a final concentration of 50 ng/ml in the presence or absence of purine analogues. The cultures were then washed free of medium and were frozen on dry ice until assay. At this time, they were scraped in 400 μl of 50 mM Tris-Cl, (pH 7.4), 1 mM EDTA, 5 mM DTT, 0.1 mM pyridoxal phosphate, and 0.5 mM L-ornithine. The extracts were sonicated on ice for 5 s and centrifuged for 15 min at 12,000 g. ODC activity was measured in supernatant fractions as described (14). [^{14}C] D,L-ornithine (specific activity 53 mCi/mmol; New England Nuclear) was used at 0.1–0.4 $\mu\text{Ci}/\text{assay}$. Released [^{14}C] was trapped in 100 μl of Protosol (New England Nuclear) absorbed on filter paper (GF/C; Whatman, Inc., Clifton, NJ) and radioactivity was assessed by liquid scintillation counting. Assays were performed using 100–300 μg of protein per sample.

Neurite Regeneration Studies

PC12 cells were pretreated with NGF for 1–2 wk and then passaged for regeneration on collagen-coated 35-mm tissue culture dishes in complete medium in the presence or absence of NGF (50 ng/ml), with or without the specified purine analogues. The cultures were then observed 1 d later and scored for proportion of neurite-bearing cell clumps as detailed elsewhere (4).

Northern Blot Analysis of c-fos mRNA

Cells were grown in 100-mm dishes and pretreated where applicable for 1 h with the appropriate final concentrations of purine analogue. NGF was added for 1 h and the plates were chilled, washed with cold buffered saline, and immediately used for isolation of total cellular RNA as previously described (6). 15 μg of total RNA per sample were separated on 1% agarose-formaldehyde gels and transferred to nitrocellulose as described (29). The nitrocellulose blots were prehybridized, hybridized with ^{32}P -labeled probe (Pst I fragment of plasmid p-fos-1; reference 9), and washed as described elsewhere (28). To confirm equality of RNA loaded, the blots were boiled and rehybridized with probe to glucose-6-phosphate dehydrogenase (28).

Protein Determination

Protein concentrations were determined by the method of Bradford (3), using reagents and protocols purchased from Bio-Rad Laboratories (Cambridge, MA) and with BSA as standard.

Results

In Vitro Inhibition of PKN by Purine Analogues

The data in Fig. 1 illustrates that PC12 cell extracts highly enriched for PKN (see Materials and Methods) catalyze the phosphorylation of histone HFI in the presence of Mn^{++} and that this activity is enhanced for extracts derived from NGF-treated cells. In assessing agents that might modulate

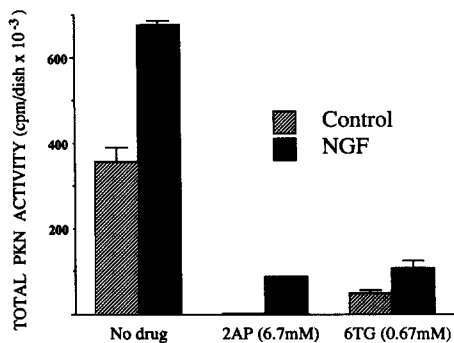


Figure 1. Basal and NGF-activated PKN activity is inhibited by purine analogues. PC12 cells were treated for 10 min with or without NGF (50 ng/ml final concentration) directly added to the culture medium. Extraction, partial purification, and assay of PKN were performed as described in Materials and Methods. Purine analogues were added directly to the kinase assay. Approximately 900 μ g of soluble protein was loaded on Mono S minicolumns. The data represent average values \pm SEM ($n = 4$).

PKN kinase activity, we tested purine analogues. A rationale for this is that one such analogue, 2-aminopurine (2-AP) has been reported to be a relatively selective protein kinase inhibitor (10). The data in Figs. 1 and 2 A and Table I show that purine analogues can effectively block PKN activity in vitro. In contrast to 2-AP and adenine which block PKN with apparent K_i values of ~ 2 mM, 6-thioguanine (2-amino-6-mercaptapurine; 6-TG), inhibited PKN with a >100 -fold lower apparent K_i of ~ 6 μ M. The corresponding nucleoside 6-thioguanosine was also an effective inhibitor with an apparent K_i ~ 40 μ M (Fig. 2 and Table I). 2-AP and 6-TG suppressed both NGF-stimulated as well as basal PKN activity (Fig. 1).

Even at very high concentrations of 2-AP or 6-TG, the kinase activity in extracts enriched for PKN was not completely inhibited (see Figs. 1 and 2). We still do not know if this is due to contamination of the PKN preparation by purine-insensitive kinases or to a resistant isoform of PKN.

Specificity with which Purine Analogues Inhibit PKN

Several different types of experiments were performed to determine the specificity with which purine analogues inhibit PKN activity. In each case, 6-TG was compared with either 2-AP or adenine. Fig. 2 B shows the effects of 6-TG and 2-AP on phosphorylation of histone HF1 in the presence of Mn^{++} by unfractionated PC12 cell extracts. In contrast to its potent blockade of partially purified PKN activity, 6-TG had no apparent effect on overall activity in the extracts, even at concentrations of up to 1 mM. On the other hand, 2-AP inhibited such activity with an IC_{50} in the range of 5 mM. When the endogenous phosphorylating activity of cell extracts was tested under the usual conditions, but in the absence of exogenous substrate (histone), neither 2-AP (7 mM), adenine (3 mM), or 6-TG (600 μ M) inhibited the overall level of phosphate incorporation or affected the pattern of protein phosphorylation (as assessed by SDS-PAGE autoradiography; data not shown). As an additional test of specificity, 6-TG and 2-AP were evaluated for their effects on protein kinase A and protein kinase C activities. At levels of up to 500 μ M, 6-TG had no effect on either kinase; 2-AP was similarly without effect at up to 5 mM (data not shown).

Purine derivatives are known to penetrate intact cells very quickly (30). Therefore the purine analogues were additionally tested for effects on phosphorylation in intact cells. The data in Fig. 3 reveal that during 3 h of incubation, 6-TG (500 μ M) had no significant effect on the overall incorporation of ^{32}P -labeled phosphate into macromolecules, whereas adenine (5 mM) inhibited this parameter by $\sim 50\%$. As illustrated by Fig. 4, the phosphoproteins labeled under these conditions were analyzed by SDS-PAGE. There were no ob-

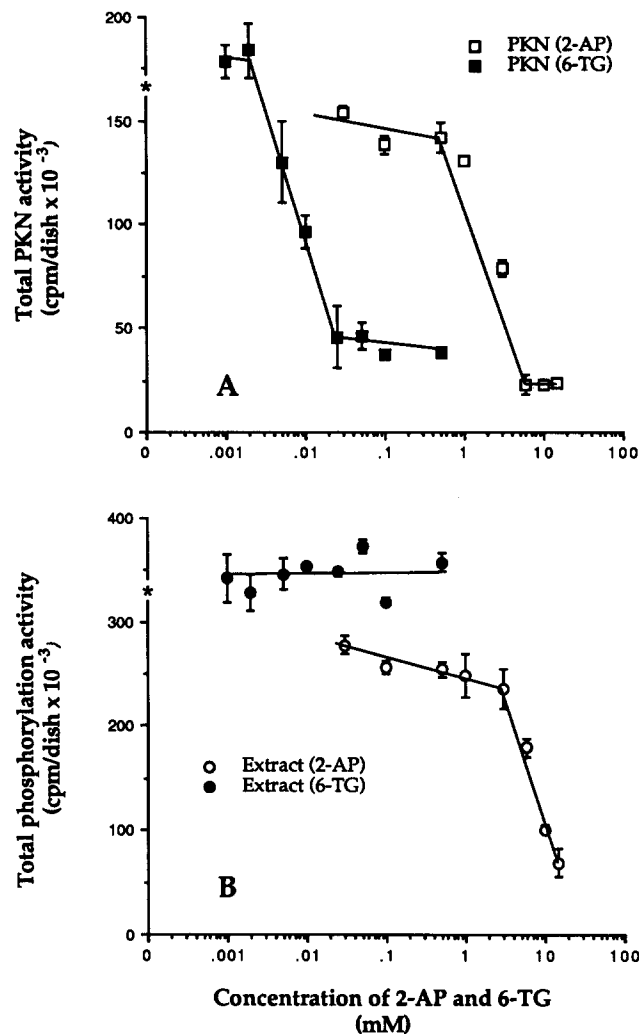


Figure 2. Effect of 2-AP and 6-TG on histone HF1 phosphorylation by PKN and by unfractionated PC12 cell extracts. Extracts were prepared from PC12 cells as described in Materials and Methods. For the data in A, PKN was partially purified by chromatography on a mini-Mono S column (~ 450 μ g of soluble protein was loaded) and equal aliquots were assayed for PKN activity in the presence of the indicated concentrations of 2-AP and 6-TG. For the data in B, aliquots of the unfractionated extract (containing approximately the same amount of protein as that used for partial purification of PKN) were assessed for phosphorylation activity under the same conditions used to measure PKN activity and in the presence of the indicated concentrations of additives. In each case the substrate was histone HF1. Values represent the total specific phosphorylation activity per sample and are expressed as means \pm SEM ($n = 4$). The asterisks on the ordinate represent the control values of PKN activity in the absence of purine analogues.

Table 1. Effect of Various Purine Analogues on PKN Activity

Analogue	IC ₅₀ for PKN inhibition
	<i>mM</i>
2-Amino-6-mercaptapurine (6-TG)	0.006
6-Thioguanosine	0.04
2-Amino-6-methylmercaptapurine	0.4
2-AP	2.0
Adenine (6-aminopurine)	2.0
Adenosine	2.5

The experiment was performed as described in the legend to Fig. 1. The compounds were added directly to the PKN enzymatic assay. Values are average of two independent experiments each performed on duplicate samples. The standard deviations were <15%.

served effects of 6-TG on the phosphoproteins that can be resolved at this level of analysis. Adenine also had few evident effects but did appear to somewhat decrease the incorporation of phosphate into a band at $\sim 50,000 M_r$. Moreover, neither 6-TG nor adenine had any observable effect on NGF-promoted changes in protein phosphorylation that occur under these conditions. This includes enhanced phosphate incorporation into MAP 1.2, a high molecular weight microtubule-associated protein (1), into tyrosine hydroxylase (18), and into several presently unidentified species of apparent molecular masses of ~ 64 , 55, and 20 kD. Two-dimensional gel electrophoresis was also performed on similar samples and the general pattern of protein phosphorylation was found to be unaffected by the presence of purine analogues (data not shown).

Effect of Purine Analogues on PKN Activation in Intact Cells

Past findings have indicated that PKN is itself activated via the actions of other protein kinases (2, 35, 36). We therefore

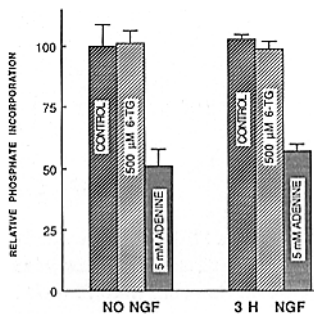


Figure 3. Effect of 6-TG and adenine on the incorporation of phosphate by PC12 cells. Sister cultures were preincubated for 1 h in the presence of either no drug (CONTROL), 500 μ M 6-TG or 5 mM adenine. They were then incubated with the same additives at 37°C for an additional 2 h in a HEPES-buffered phosphate-free saline in the presence of 100 μ Ci/ml of [³²P]orthophosphate, with or without NGF. After incubation, the cells were washed to remove external phosphate and were harvested in 500 μ l of SDS sample buffer. Aliquots (50 μ l) were assessed (by liquid scintillation counting) for radioactivity present either in the total extract or in TCA-precipitable material. In each case, the values of the latter were corrected for total phosphate uptake in the cells by normalizing to radioactivity in the total extract. TCA-precipitable counts accounted for $\sim 5\%$ of the total radioactivity in the extracts ($\sim 10^6$ cpm/culture). Values are expressed as normalized TCA-precipitable counts per culture relative to that in control, nontreated cultures and are given as averages \pm SEM ($n = 3$). Comparable results were achieved in three independent experiments.

investigated the ability of purine analogues to affect activation of PKN by NGF in intact cells. PC12 cells were treated for 10 min with or without NGF in the presence or absence of 10 mM 2-AP or 500 μ M 6-TG (both concentrations are

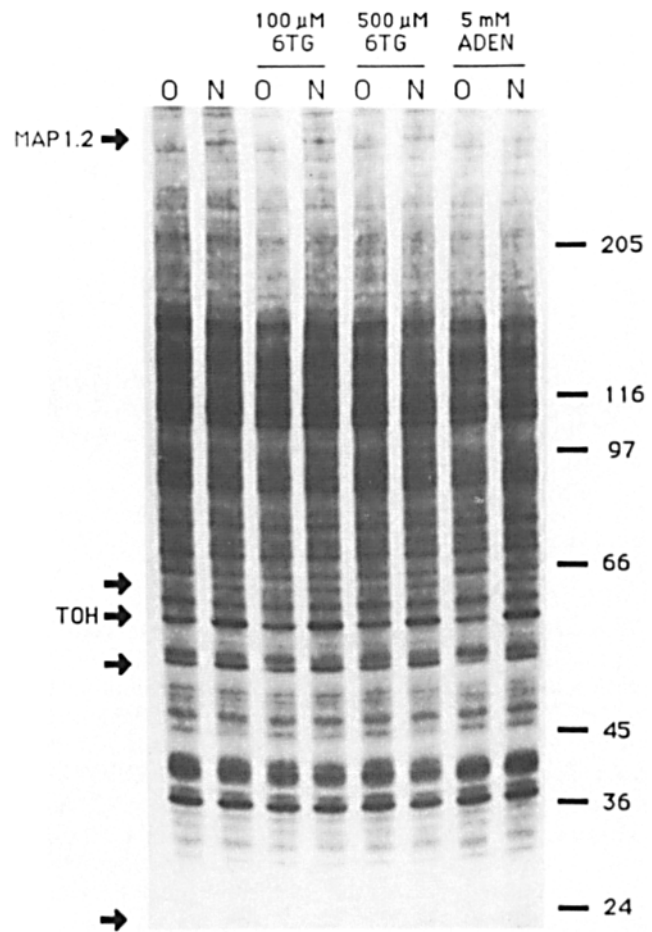


Figure 4. Effects of 6-TG and adenine on the patterns of basal and rapidly NGF-regulated protein phosphorylation in PC12 cells. Cultures were treated as described in the legend of Fig. 3 except that cells treated with 100 μ M 6-TG were also tested and harvesting was in 400 μ l of sample buffer. Aliquots containing equal numbers of cpm (50,000) were subjected to SDS-PAGE and autoradiography as described in Materials and Methods. Numbers on the right represent the position of molecular mass standards (given as $M_r \times 10^{-3}$).

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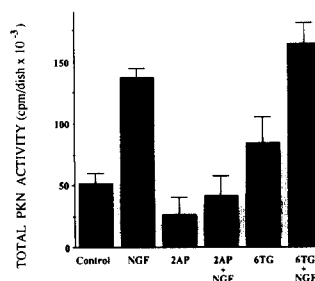


Figure 5. Effect of 2-AP and 6-TG on NGF-promoted activation of PKN in intact PC12 cells. Replicate cultures were treated for 10 min with or without NGF and in the presence or absence of 10 mM 2-AP or 500 μ M 6-TG. The cells were then rapidly washed and extracts were prepared and used for the partial purification and assay of PKN as

described in Materials and Methods. Approximately 200 μ g of soluble protein was loaded on each Mono S minicolumn. Values represent the total specific phosphorylation activity per sample and are expressed as means \pm SEM ($n = 4$).

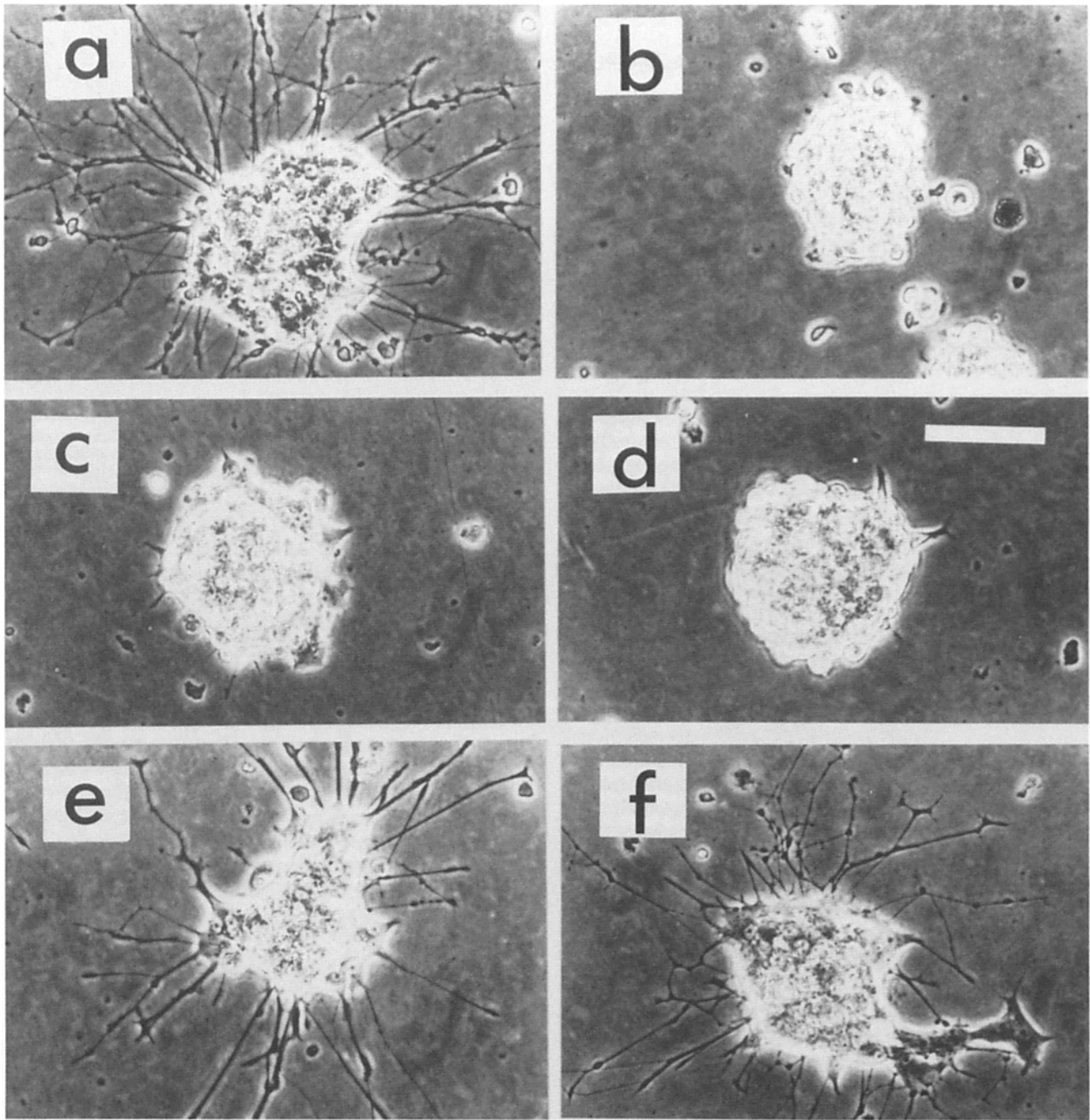


Figure 6. NGF-dependent neurite regeneration by PC12 cells: inhibition by purine analogues and reversibility of the effect. PC12 cells were pretreated for 10 d with NGF (50 ng/ml) and then passaged for regeneration on collagen-coated 35-mm tissue culture dishes in RPMI 1640 medium containing 1% horse serum and in the presence or absence of NGF (50 ng/ml), with or without purine analogues. (a–d) Cells are shown 24 h after they were replated for regeneration; (a) in the presence of NGF; (b) without NGF; (c) with NGF and 30 μ M 6-thioguanine; (d) with NGF and 5 mM adenine. After the cells were cultured for 24 h with NGF in the presence of purine analogues, they were rinsed and the medium was replaced with fresh medium containing only NGF. Cells are shown 2 d after washout of 30 μ M 6-thioguanine (e) or of 5 mM adenine (f).

above the IC_{50} values for in vitro inhibition of PKN). The cultures were then rapidly washed free of the drugs and assessed for PKN activity. Under these conditions, since the purine analogues were removed and any remaining material was highly diluted during the partial purification and assay of the enzyme, any noted effects were due to interference with activation of PKN in situ, rather than due to direct inhi-

bition during the assay. The data in Fig. 5 show that 2-AP suppressed activation of PKN by NGF in intact cells while 6-TG did not. 6-TG failed to block PKN activation even after 4 h of preincubation with the cells (not shown). In contrast, when the NGF-activated PKN was partially purified from cells that had been incubated for 4 h with 6-TG, this activity was inhibitable in vitro by both 2-AP and 6-TG (data not shown).

One interpretation of such results is that 2-AP, in addition to directly inhibiting PKN, also blocks a phosphorylation event in the NGF-regulated pathway that leads to PKN activation. In contrast 6-TG is more specific for PKN and does not block the activation pathway. This interpretation is consistent with the above-described differences in apparent specificity of 2-AP and 6-TG for inhibition of histone phosphorylation by unfractionated cell extracts.

In light of the effects of purine analogues on PKN activity, these compounds were assessed for their effects on various biological responses to NGF.

Effect of Purine Analogues on Neurite Regeneration

When neuronally differentiated PC12 cells are mechanically deprived of their neurites and then replated, they undergo NGF-promoted neurite regeneration within 1 d (Fig. 6). This rapid effect does not require RNA synthesis (4). Fig. 6 shows the effect of inclusion of 30 μ M 6-TG or 5 mM adenine during such regeneration. In both cases, neurite outgrowth was suppressed, even though the cells appeared well-attached to the substrate and in good condition. Further evidence for the latter was that the effect was reversible; washout of the drugs was followed within 1–2 d by the appearance of normal neurites (Fig. 6).

Fig. 7 shows a dose-response curve of the effect of various purine analogues on PC12 cell neurite regeneration. Table II summarizes the apparent IC_{50} values for these and additional analogues. As in the case of PKN inhibition, 6-TG is by far the most effective compound with an IC_{50} of ~ 3 μ M. 2-AP and adenine, as well as several other analogues, exhibit IC_{50} values in the range between 1 and 10 mM. Both purine and guanine were without effect at the highest concentrations of each that could be tested (10 and 0.5 mM, respectively). Finally, 2-amino-6-methylmercaptapurine, similar to its effect on PKN inhibition in vitro (Table I), had an intermediate activity with an IC_{50} of ~ 200 μ M (Table II).

Effects of Purine Analogues on Induction of ODC Activity in Intact PC12 Cells

NGF treatment promotes a large (>10-fold) induction of ODC activity within 4–6 h of addition to PC12 cell cultures (14). In contrast to NGF-promoted neurite regeneration, this action is dependent on RNA synthesis (5) and appears to be

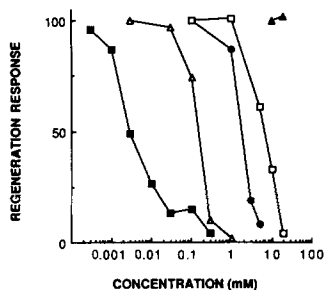


Figure 7. Dose-response relationships for effects of purine analogues on NGF-dependent neurite regeneration. PC12 cells were pretreated for 10 d with NGF (50 ng/ml) and then passaged for regeneration on collagen-coated 35-mm tissue culture dishes in RPMI medium containing 1% horse serum with NGF (50 ng/ml), with or without the indicated

concentrations of purine analogues. Neurite-bearing cell clumps were scored at 18–24 h after replating. Values are expressed relative to the total proportion of cell clumps regenerating neurites in the absence of drugs (80–95%). At least 100 cell clumps were scored per sample. \bullet , adenine; \blacksquare , 6-TG; \square , 2-AP; \triangle , 6-MTG; \blacktriangle , purine.

Table II. Effect of Various Purine Derivatives on PC12 Cell Neurite Regeneration

Drug	IC_{50} for neurite regeneration
	mM
2-Amino-6-mercaptapurine (6-TG)	0.003
2-Amino-6-methylmercaptapurine	0.2
6-Cyanopurine	1.0
Adenine (6-aminopurine)	2.0
6-Mercaptapurine	2.5
Theophylline	2.5
2, 6-Diaminopurine	3.0
2-AP	7.0
Guanine (2-amino-6-hydroxypurine)	>0.5
2-Hydroxy-6-Mercaptapurine (6-thioxanthine)	>10.0
Purine	>20.0

PC12 cell neurite regeneration was carried out as described in the legend to Fig. 7 with or without various concentrations of the above compounds. For each compound, dose-response curves were generated and these were used to determine the IC_{50} values. For guanine, 6-thioxanthine and purine, values given were the highest concentrations that could be tested due to limitation of solubility.

due to enhanced transcription of the ODC gene (12). Fig. 8 shows the effect of NGF added along with various concentrations of 6-TG or 2-AP. 6-TG inhibited the induction of ODC activity with what appears to be a biphasic dose-response curve. About 70% of the induction was blocked with an apparent IC_{50} <10 μ M. The remaining induction was blocked only at concentrations >1 mM. 2-AP also showed a biphasic dose response curve. In this case, the induction was consistently somewhat enhanced by concentrations of up to 5 mM and was effectively blocked at 10 mM. Adenine and diaminopurine also effectively suppressed the induction of ODC. A 60% inhibition was observed with 5 mM adenine and total inhibition with 10 mM diaminopurine. Uric acid, the degradation product of purine derivatives, was without effect at the highest concentrations that could be tested (500 μ M).

Effect of Purine Analogues on Induction of *c-fos* mRNA

Another early transcription-dependent action of NGF is the induction of *c-fos* (12). Zinn et al. (41) have shown that 2-AP suppresses the induction of this proto-oncogene in human MG63 osteosarcoma cells. Fig. 9 illustrates the effects of 2-AP and 6-TG on *c-fos* mRNA levels in PC12 cells treated with NGF. As in MG63 cells, 2-AP (10 mM) inhibits the increase in *c-fos* mRNA. For the experiment shown in Fig. 9, there was a 70% blockade of induction. In three additional independent experiments (not shown), inhibition appeared to be nearly complete. In contrast, 6-TG (100 μ M in Fig. 9) did not inhibit the induction of *c-fos* mRNA by NGF, and in fact, appeared to enhance this effect. Other experiments (not shown) revealed that, as in the presence of NGF alone (12), the *c-fos* mRNA induction was transient in the presence of 6-TG and NGF, peaking at ~ 1 h and being undetectable by 4 h of treatment.

Discussion

The purpose of the present work was to investigate how NGF promotes and coordinates the diverse group of responses that

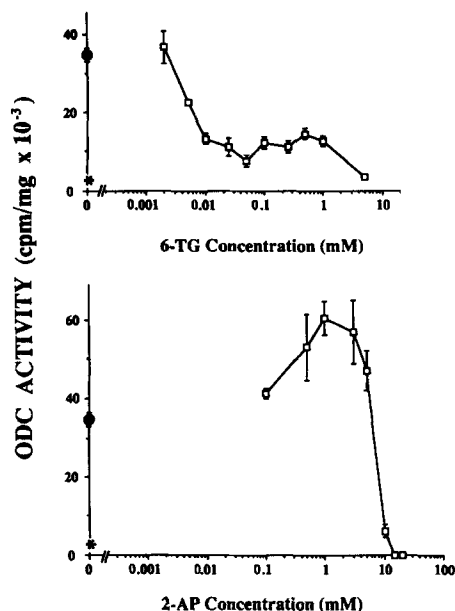


Figure 8. NGF-induced ODC activity is inhibited by 2-AP and 6-TG. PC12 cells were cultured on collagen-coated 35-mm tissue culture dishes in RPMI 1640 medium containing 1% horse serum. NGF (50 ng/ml) was added for 6 h in the presence or absence of the indicated concentrations of 2-AP or 6-TG. [¹⁴C]D,L-ornithine was used at 0.45 μ Ci/assay. Values are averages of determinations on replicate cultures \pm SEM ($n = 3$). The asterisks represent the value of ODC activity in PC12 cells not cultured with NGF and the closed circle represents the activity without added analogue.

it elicits in its target cells. To this end, we have used purine analogues to modulate and dissect specific effects of NGF.

Our data show that the purine analogue 6-TG can effectively block certain responses to NGF while sparing others. For example, neurite regeneration was suppressed by 6-TG while several additional transcription-independent NGF-promoted responses, namely activation of PKN itself and enhanced phosphorylation of specific proteins, were apparently unaffected. This was also the case for transcription-dependent actions of NGF; 6-TG suppressed the induction of ODC activity but did not inhibit the induction of *c-fos* mRNA.

One mechanism that could account for the effects of 6-TG on NGF responses that is consistent with our observations is interference with the activity of PKN. Thus, 6-TG interferes with PKN activity in a cell-free assay and does so with a potency similar to that with which it blocks biological responses in intact cells. Moreover, at the levels of analysis used here, 6-TG did not detectably affect protein kinase activities other than PKN.

Aside from its effects on PKN, there are of course additional cellular actions of 6-TG that merit consideration in light of our present observations. Most notably, 6-TG is a potent cytotoxic agent. However, the toxic effects of this compound on PC12 cells were not detectable before 2–3 d of treatment, that is, well beyond the appearance of the parameters that were assessed. Also, in the case of neurite regeneration, in which treatment with the drug was for 1 d, the inhibitory effects were reversed after washout. Perhaps related to its lethal actions, 6-TG is incorporated into cellular DNA and alters the synthesis and function of RNA and DNA (40).

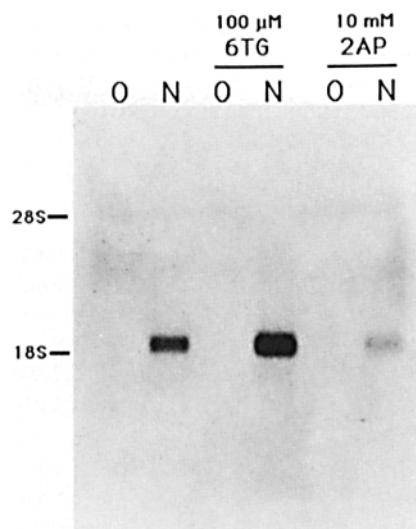


Figure 9. Effect of purine analogues on NGF-dependent induction of *c-fos*. Cells were grown in 100-mm dishes and pretreated where applicable for 1 h with the appropriate final concentrations of purine analogue. NGF was added for 1 h and the cells were immediately used for isolation of total cellular RNA. 15 μ g of total RNA per sample were separated on 1% agarose-formaldehyde gels and transferred to nitrocellulose. The nitrocellulose blots were pre-hybridized, hybridized with ³²P-labeled probe, washed, and autoradiographed, all as described in Materials and Methods.

Such effects would, however, seem unlikely to be directly involved in the blockade of NGF responses. Neurite regeneration is independent of RNA synthesis (4) while at least one transcription-dependent action of NGF, the induction of *c-fos* mRNA, is not blocked by 6-TG.

Our findings also revealed differences in effect between 6-TG and several other purine analogues. In contrast to 6-TG, 2-AP blocked NGF-promoted activation of PKN and, in agreement with earlier findings (41), suppressed induction of *c-fos* mRNA. Studies of the specificities with which these compounds inhibit cellular protein kinase activity also revealed marked differences. At concentrations similar to those at which they affected PKN activity, 2-AP and adenine partially inhibited phosphorylation of histone by cell extracts; 6-TG was ineffective in this respect. Finally, 2-AP and several other purine analogues were two to three orders of magnitude less potent than 6-TG in inhibition of PKN activity in vitro, and in suppression of neurite regeneration and ODC induction. These observations are consistent with the possibility that compounds such as 2-AP and adenine exert at least some of their NGF-inhibitory actions by interfering both with the activation and with the activity of PKN. Additional suppressive actions of these analogues not shared with 6-TG could be due to their lesser specificity as protein kinase inhibitors. For instance, earlier findings have suggested that PKN might itself be activated by phosphorylation via another NGF-activated protein kinase (2, 35, 36). It is thus conceivable that 2-AP interferes with this NGF-regulated kinase.

Several other agents have been found to interfere with responses to NGF and it is of interest to compare their actions with the effects of purine analogues. Inhibitors of cellular *trans*-methylation, all of which are adenosine analogues, interfere with neurite outgrowth and ODC induction (37).

However, in contrast to 6-TG, these also appear to suppress all other responses to NGF including rapid tyrosine hydroxylase phosphorylation (37) and induction of *c-fos* gene transcription (12). The alkaloid protein kinase inhibitor K-252a similarly differs from purine analogues in that it blocks all known responses of PC12 cells to NGF (20, 22). Forskolin and cholera toxin have been found to block NGF-dependent neurite regeneration (17), but differ from purine compounds in that they also suppress tyrosine hydroxylase phosphorylation (17) and induce ODC (38). Lithium ion is an additional agent that suppresses NGF-promoted neurite outgrowth and selectively interferes with the phosphorylation of chordin microtubule-associated proteins (5). However, this ion does not block PKN activity in vitro (Volonté, C., unpublished results).

As noted above, such agents as *trans*-methylation inhibitors and K252-a block all responses to NGF whereas other compounds such as purine analogues affect only selective NGF actions. This suggests a single initial pathway in the NGF mechanism followed by a set of divergent pathways. In particular, 6-TG appears to dissect out a pathway or set of pathways in the NGF mechanism that play necessary roles in neurite regeneration and in ODC induction, but that seem distinct from those that lead to *c-fos* gene activation and to rapid phosphorylation of several intracellular proteins.

We have suggested that PKN is activated by NGF-promoted phosphorylation, and that 6-TG exerts its inhibitory actions on NGF responses through blockade of PKN activity. One may further conceive of a cascade of branching pathways that are initiated after NGF binding and that this cascade is driven, at least in part, by regulation of various protein kinases. This is consistent with the large variety of different cellular responses that are triggered by NGF. Activators of protein kinases such as PKA and PKC have been reported to mimic some, but not other responses of cells to NGF (25). This might occur if PKA and PKC can in turn activate certain, but not all, pathways of the NGF mechanism. For instance, both of these kinases activate PKN (2, 35, 36). In this light, it is of interest that purine analogues block induction of ODC brought about by exposure of PC12 cells to a cAMP derivative (Volonté, C., and D. Loeb, unpublished data).

Our studies have examined the effects of purine analogues on NGF responses in a cell culture system. One might further raise the possibility that excessive accumulation of purine derivatives could, with detrimental consequences, also interfere with trophic factor actions in the intact organism.

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