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
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 (lysin 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 (7.9 ng/ml) was added to the medium in the presence or absence of purine analogues. The cultures were then washed free of medium and were frozen on dry ice until assay. 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 harvested, washed, and centrifuged at 4°C for 15 min at 6000 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 centrifuged 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 centrifuged 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 centrifuged 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 centrifuged for 15 min at 60,000 rpm in an ultracentrifuge (model TL-100; SN-941 rotor; Beckman Instruments, Inc., Palo Alto, CA).

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 MnCl2, 2 mM PMSE, 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 centrifuged 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 centrifuged 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 centrifuged 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 centrifuged for 15 min at 60,000 rpm in an ultracentrifuge (model TL-100; SN-941 rotor; Beckman Instruments, Inc., Palo Alto, CA).

Assay of cAMP-dependent Protein Kinase (PKA) and Ca2+/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 PMSE, 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, 0.1 µl of cAMP (7, 10 µM cAMP). 1 µCi of [γ-32P]ATP (3,000 Ci/mmol; New England Nuclear) in ATP (50 µM final concentration) was added and the phosphorylation reaction was started by adding the kinase assay (10 µl) and the radioactive 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 PMSE, 100 KIU/ml Trasylol) and the phosphorylation reaction was performed in buffer C, ± 1.5 mM CaCl2 and with 5 µg/ml phosphatidyserine and 0.5 µg/ml diol (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 µCi/ml of [32P]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 then 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 harvested, washed, and centrifuged at 4°C for 15 min at 12,000 g. ODC activity was measured in supernatant fractions as described (14). [1-14C]N,l-ornithine (specific activity 53 mCi/mmol; New England Nuclear) was used at 0.1-0.4 µCi/assay. 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 then hybridized with [32P]-labeled probe (Pst I fragment of plasmid p-fos-I; reference 9), and washed as described elsewhere (28). To confirm equality of RNA loaded, the blots were then hybridized 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 Mn2+ and that this activity is enhanced for extracts derived from NGF-treated cells. In assessing agents that might modulate...
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 \( \mu \)M) had no significant effect on the overall incorporation of \(^{32}\)P-labeled phosphate into macromolecules, whereas adenine (5 \( \mu \)M) 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 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 \( \mu \)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 \( \sim 2 \) mM, 6-thioguanine (2-amino-6-mercaptopurine; 6-TG), inhibited PKN with a \( >100\)-fold lower apparent \( K_i \) of \( \sim 6 \) \( \mu \)M. The corresponding nucleoside 6-thioguanosine was also an effective inhibitor with an apparent \( K_i \) of \( \sim 40 \) \( \mu \)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\(^{2+}\) 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 endogeneous 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 \( \mu \)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 \( \mu \)M, 6-TG had no effect on either kinase; 2-AP was similarly without effect at up to 5 mM (data not shown).
Table 1. Effect of Various Purine Analogues on PKN Activity

| Analogue                          | IC₅₀ for PKN inhibition (mM) |
|----------------------------------|-----------------------------|
| 2-Amino-6-mercaptopurine (6-TG)  | 0.006                       |
| 6-Thioguanosine                  | 0.04                        |
| 2-Amino-6-methylmercaptopurine   | 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%.

Table 2. Effects of Purine Analogues on PKN Activity in Intact Cells

Past findings have indicated that PKN is itself activated via the actions of other protein kinases (2, 35, 36). We therefore 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 µM 6-TG (both concentrations are NGF NGF described in Materials and Methods. Numbers on the right represent the position of molecular mass standards (given as M⁰ x 10⁻³).

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 µM 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 µCi/ml of [³²P]orthophosphate, with or without NGF. After incubation, the cells were washed to remove external phosphate and were harvested in 500 µl of SDS sample buffer. Aliquots (50 µl) 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 ~5% of the total radioactivity in the extracts (~10⁶ cpm/culture). Values are expressed as normalized TCA-precipitable counts per culture relative to that in control, nontreated cultures and are given as averages ± SEM (n = 3). Comparable results were achieved in three independent experiments.

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 µM 6-TG were also tested and harvesting was in 400 µl 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 M⁰ x 10⁻³).

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 µM 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 µg of soluble protein was loaded on each Mono S minicolumn. Values represent the total specific phosphorylation activity per sample and are expressed as means ± SEM (n = 4).
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 μM 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 μM 6-thioguanine (e) or of 5 mM adenine (f).

above the IC₅₀ 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 inhibition 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 unfractonated 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 Analogue 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 μM 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 IC₅₀ values for these and additional analogues. As in the case of PKN inhibition, 6-TG is by far the most effective compound with an IC₅₀ of ~3 μM. 2-AP and adenine, as well as several other analogues, exhibit IC₅₀ 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-methylmercaptopurine, similar to its effect on PKN inhibition in vitro (Table I), had an intermediate activity with an IC₅₀ of ~200 μM (Table II).

**Effects of Purine Analogue 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 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 IC₅₀ <10 μM. 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 dianinopurine also effectively suppressed the induction of ODC. A 60% inhibition was observed with 5 mM adenine and total inhibition with 10 mM dianinopurine. Uric acid, the degradation product of purine derivatives, was without effect at the highest concentrations that could be tested (500 μM).

**Effect of Purine Analogue 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 μM 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

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

| Drug                                      | IC₅₀ for neurite regeneration (mM) |
|-------------------------------------------|----------------------------------|
| 2-Amino-6-mercaptopurine (6-TG)           | 0.003                            |
| 2-Amino-6-methylmercaptopurine            | 0.2                              |
| 6-Cyanoptopurine                         | 1.0                              |
| Adenine (6-aminopurine)                  | 2.0                              |
| 6-Mercaptopurine                         | 2.5                              |
| Theophylline                              | 2.5                              |
| 2, 6-Diaminoptopurine                    | 3.0                              |
| 2-AP                                     | 7.0                              |
| Guanine (2-amino-6-hydroxypurine)        | >0.5                             |
| 2-Hydroxy-6-Mercaptopurine (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 IC₅₀ values. For guanine, 6-thioxanthine and purine, values given were the highest concentrations that could be tested due to limitation of solubility.
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. [3-14C]1-ornithine was used at 0.45 μCi-assay. Values are averages of determinations on replicate cultures ± 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.

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 μg of total RNA per sample were separated on 1% agarose-formaldehyde gels and transferred to nitrocellulose. The nitrocellulose blots were prehybridized, 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 chartin 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 AMP 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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