Purine analogues were used to probe the mechanism by which nerve growth factor (NGF) and other agents regulate cellular ornithine decarboxylase (ODC) activity. Exposure of cultured rat pheochromocytoma PC12 cells to NGF causes a 10-50-fold induction of ODC activity within 4–6 h. We recently found that purine analogues block this induction as well as other, but not all, actions of NGF and have provided evidence that the inhibitory actions of the analogues may be due in part to the suppression of an NGF-activated protein kinase activity (Volonté, C., Rukenstein, A., Loeb, D. M., and Greene, L. A. (1989) J. Cell Biol. 109, 2395–2403). The present results show that the purine analogues also suppress the induction of ODC mRNA. One of the analogues used was 6-thioguanine (6-TG). Although 6-TG was effective when applied simultaneously with NGF, if NGF was administered for as little as 1–3 min before 6-TG, ODC induction was unimpaired. This suggests that 6-TG blocks an early step in the NGF mechanism, and that once this step is triggered, the ODC induction pathway is no longer sensitive to this analogue. In contrast, another purine analogue, 2-aminopurine (2-AP), effectively inhibited ODC induction even if applied only during the last hour of a 5-h exposure to NGF. It is hypothesized that this increased period of sensitivity to 2-AP may be due to its broader range (as compared to 6-TG) as an inhibitor of protein kinase activities. Epidermal growth factor (EGF) and cAMP derivatives also induce ODC activity in PC12 cells, and these effects were suppressed by 6-TG and 2-AP at concentrations similar to those that affect responses to NGF. However, short term (<30 min) pretreatment with EGF or a cAMP derivative did not protect induction of ODC activity by these agents from inhibition by 6-TG. This suggests that there are both convergent and divergent elements in the mechanistic pathways used by NGF, cAMP analogues, and EGF to induce ODC.

Ornithine decarboxylase (EC 4.1.1.17) (ODC) catalyzes the formation of putrescine from ornithine, the first step in the biosynthesis of polyamines (1). This pathway is active during the differentiation of several tissues and cell lines (3). Numerous studies have confirmed the role of ODC in the regulation of macromolecular events such as transcription, translation, and DNA synthesis (4–6). Recently, attention has been focused on the possible participation of ODC in postreplicative events in the nervous system such as axonogenesis, synaptogenesis, cell death, and regrowth after axonal injury (7–10).

Nerve growth factor (NGF) (11) plays a major role in the growth, differentiation, and survival of sympathetic and sensory neurons, and NGF and its receptors are also present in the central nervous system (12–15). ODC activity has been shown to be markedly stimulated in rat superior cervical ganglia (16), rat brain (17), and cultured rat PC12 pheochromocytoma cells (18–20) by NGF. The PC12 cell line (21) has proved useful for studying the mechanism of action of NGF as well as the pathways involved in ODC regulation (18, 22, 23). NGF causes PC12 cells to change their phenotype from that of immature chromaffin-like cells to that of sympathetic neurons. Within 4–6 h of NGF exposure, PC12 cells undergo a 10–50-fold induction of ODC activity: this appears to be regulated, at least in part, at the transcriptional level (20, 24). In addition to NGF, several other agents, including cAMP derivatives and epidermal growth factor (EGF), induce ODC activity in PC12 cells (19, 22).

We recently demonstrated that purine analogues can differentially inhibit certain, but not other, actions of NGF in PC12 cells (25, 26). For instance, these analogues suppress NGF-stimulated neurite regeneration and induction of ODC activity, but do not block NGF enhanced phosphorylation of several proteins or support of neuronal survival. We also noted differences among the purine analogues: 6-thioguanine (6-TG) exhibited an inhibitory activity in the micromolar range while most of the other analogues, exemplified by 2-aminopurine (2-AP), had activities in the millimolar range. Also, while 2-AP blocked the NGF-stimulated induction of c-fos mRNA, 6-TG did not. We further noted that the purine analogues inhibited the in vitro activity of an NGF-activated protein kinase (27) and did so with relative potencies comparable to those at which they blocked NGF-promoted neurite regeneration and ODC induction in intact PC12 cells (25).

In the present study, we have used purine analogues to address several issues regarding the pathways by which NGF and other agents regulate ODC induction in PC12 cells. One is whether the suppression of NGF-induced ODC activity by purine analogues occurs at a pre- or post-translational level. A second issue regards the point in the induction pathway at which the purine analogues work. That is, since the up-regulation of ODC occurs at a pre- or post-translational level, are the analogues blocking an early or late step in this induction process? A third is whether the analogues can...
distinguish between the mechanistic pathways by which several agents in addition to NGF induce ODC.

**EXPERIMENTAL PROCEDURES**

**Materials—**Purine analogues, were purchased from Sigma. Stock solutions (1-100 mM) were prepared in RPMI 1640 medium or in water (both adjusted to pH 7.4) (25). 8-(4-Chlorophenylthio)-cAMP (CPT-cAMP) was purchased from Sigma. EGF was kindly provided by Dr. F. R. Maxfield (Columbia University, Dept. of Pathology).

**Cell Culture—**Stock cultures of PC12 cells were maintained as previously described on collagen-coated culture dishes in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum (21). For experiments, cells were subcultured in collagen-coated 35-mm dishes and maintained overnight in RPMI medium containing 1% horse serum (18). Where specified, mouse submaxillary NGF (28) and/or various purine analogues were directly added to the cultures from concentrated stocks.

**Assay of Ornithine Decarboxylase Activity—**This was measured by methods slightly modified from those previously described (18). NGF was added to replicate cultures for 5 h at a final concentration of 50 ng/ml (2 mM) in the presence or absence of purine analogue. The cultures were then washed free of media and were frozen on dry ice until assay. At this time, they were harvested in 400 μl of ice-cold 50 mM Tris-Cl (pH 7.4), 1 mM EDTA, 5 mM dithiothreitol, 0.1 mM pyridoxal phosphate, and 0.5 mM l-ornithine. The extracts were sonicated for 5 s and centrifuged at 4 °C for 15 min at 12,000 g. ODC activity was measured in supernatant fractions as described (18). l-[1-14C] Ornithine (specific activity 50 mCi/mmol, DuPont-New England Nuclear) was used at 0.1-0.4 μCi/assay. Released [14CO2] was trapped in 100 μl of Protosol (DuPont-New England Nuclear) absorbed on filter paper (Whatman GF/C), and radioactivity was assessed by liquid scintillation counting. Assays were performed using 100-300 μg of protein per sample. Protein concentrations were determined by the method of Bradford (29), using reagents and protocols purchased from Bio-Rad and with bovine serum albumin as standard.

**Northern Blot Analysis of ODC 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, and the cultures were incubated for an additional 2-4 h. In some experiments, the analogues were applied simultaneously with NGF or after specified intervals. The cultures were then chilled on ice, washed with cold buffered saline, and immediately used for isolation of total cellular RNA as previously described (30). Ten μg of total RNA per sample were separated on 1.2% agarose-formaldehyde gels and transferred to nylon membranes. The blots were prehybridized, hybridized with 32P-labeled probe (PstI fragment of plasmid ODC (32)), and washed as described elsewhere (33). To confirm equality of RNA loaded, the blots were boiled and rehybridized with probe to glyceraldehyde-3-phosphate dehydrogenase (34).

**RESULTS**

**Induction of ODC Activity by NGF Is Inhibited by Purine Analogues—**It is known from previous studies that NGF, through a transcription-dependent process, causes at least a 10-fold induction of ODC activity within 4-6 h of addition to PC12 cell cultures (18, 19). We have reported (25) that 6-TG and 2-AP inhibit this induction with IC50 values of about 5-10 μM and 5-10 mM, respectively. Fig. 1 confirms these findings and shows that several additional purine analogues substantially reduce ODC induction when administered to the cells simultaneously with NGF. Uric acid, the degradation product of purines, had no effect at the highest concentration that could be tested (0.5 mM). Also, the less permeant nucleoside derivatives of 6-thioguanine and adenine (6-thioguanosine and adenosine, respectively) were ineffective in suppressing the induction of ODC activity.

To test the level at which the purine analogues inhibit NGF-promoted ODC induction, PC12 cultures (±NGF and ±6-TG or 2-AP for various times) were assessed for their relative levels of ODC mRNA. The drugs were applied 1 h prior to NGF and were present throughout the incubation. As previously reported, two ODC mRNA species were resolved, apparently due to the alternate use of two polyadenylation signals (35). The data in Fig. 2 show that 2-AP and 6-TG effectively inhibited the induction of both ODC mRNAs. Comparable results were achieved if the drugs were added simultaneously with NGF. Both analogues also suppressed the basal levels of ODC message in NGF-untreated cultures. A dose-response experiment (data not shown) established that 6-TG inhibited ODC mRNA induction with an IC50 of less than 10 μM and caused a 90% inhibition at 100 μM.

**Effects of Purine Analogues on ODC Induction**

**FIG. 1.** Inhibition of NGF-stimulated ODC induction by purine analogues. PC12 cells were cultured overnight on collagen-coated 35-mm tissue culture dishes in RPMI 1640 medium containing 1% horse serum. NGF (50 ng/ml final concentration) was added for 5 h in the presence or absence of different concentrations of purine or nucleoside analogues. 2-AP = 10 mM 2-aminopurine; 6-TG = 0.5 mM 6-thioguanine; Ad = 10 mM adenosine; DAP = 10 mM 2,6-diaminopurine; UA = 0.5 mM uric acid; 6-TGs = 0.5 mM 6-thioguanosine; Ads = 10 mM adenosine; 2-A,6-MMP = 3.3 mM 2-amino-6-methylmercaptopurine. ODC activity was assessed as described under "Experimental Procedures" using l-[l-14C]ornithine at 0.1 μCi/assay. Values are averages of determinations on replicate cultures ± S.E. (n = 3 to 6). The values on the ordinate are given as picomoles of CO2 released/mg of protein/h. The value of ODC activity for PC12 cells never exposed to NGF was 38 ± 4 pmol/mg of protein/h.

**FIG. 2.** Inhibition of NGF-induced ODC mRNA levels by purine analogues. Cells were grown in 100-mm dishes and pretreated where applicable for 1 h with 100 μM 6-TG or 10 mM 2-AP (final concentrations). NGF was added for different times, and the cells were then immediately used for isolation of total cellular RNA. The dashes in the figure caption represent no treatment with NGF and/or with the indicated purine analogues. Samples each contained 10 μg of RNA and were subjected to electrophoresis on 1.2% agarose-formaldehyde gels and transferred to nylon membranes. The blots were prehybridized, hybridized with 32P-labeled probe, washed, and autoradiographed, all as described under "Experimental Procedures."
lular concentrations within 30 s (36). For the experiment shown in Fig. 3, cultures were first treated with NGF and then, after various intervals (1-290 min), additionally with 500 μM 6-TG. After a total of 5 h, the cultures were harvested and assayed for ODC activity. The data in Fig. 3 show that when the cultures were pretreated with NGF for approximately 3 min, they were no longer vulnerable to inhibition of ODC induction by 6-TG. That is, even though 6-TG was present during the last 297 min of the 310 min treatment, induction of ODC activity was unaffected. A comparable experiment revealed that induction of ODC mRNA was also unimpaired under such conditions (data not shown). In some experiments, 1 min of NGF pretreatment was sufficient to prevent the inhibitory action of 6-TG on induction of ODC activity. Preincubation with 6-TG for 60 min was also evaluated and showed no significant difference from simultaneous application with NGF (data not shown).

Contrasting Effects of 6-TG and 2-AP in Inhibition of ODC Activity—A comparable set of NGF pretreatment experiments was carried out with 10 mM 2-AP. In contrast to the case of 6-TG, when 2-AP addition was delayed for up to 4 h, its capacity to inhibit NGF-stimulated induction of ODC activity (Fig. 4) and of ODC mRNA (data not shown) was undiminished. As shown in Fig. 4, there was partial inhibition of induction of ODC activity if 2-AP was present during the last half-hour of NGF treatment, and no significant effect if it was added during the last 10 min of incubation. The addition of 2-aminopurine (up to 20 mM) or 6-thioguanine (up to 10 mM) directly to the ODC assay had no significant effect on enzymatic activity (data not shown).

Effects of Purine Analouges on Induction of ODC Activity by Various Agents—Several agents in addition to NGF can induce ODC activity in PC12 cells (19, 22). This appears to occur via at least several distinct pathways (23). Fig. 5 compares the effect of a permeant cAMP analogue (CPT-cAMP), EGF, and NGF on PC12 cell ODC activation and the sensitivity of these activations to 500 μM 6-TG or 10 mM 2-AP. All three agents induced ODC activity, and these effects were substantially suppressed by the purine analogues.

Fig. 6 shows a dose-response relationship for the inhibition of CPT-cAMP induced ODC activity by 6-TG and 2-AP. As with NGF (25), inhibition by 6-TG occurred in the micromolar range, while inhibition by 2-AP was most pronounced at millimolar concentrations. However, there were several differences with our previous observations of NGF-induced ODC. First, with NGF, there was a consistent 60-70% potentiation of ODC induction by 2-AP in the range 0.3-5 mM; this
Effects of Purine Analogues on ODC Induction

was never observed for induction by CPT-cAMP. Second, while inhibition by 6-TG reached a plateau value above 50 
µM for both NGF and CPT-cAMP, with the former but not the latter, 5 mM 6-TG brought about a further suppression of 
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the induction of ODC by NGF and CPT-cAMP was in the 
time of sensitivity to 6-TG. While 1–3 min of NGF pretreat-
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produce the same effect (Fig. 7A). Since PC12 cells can 
respond to externally applied CAMP derivatives within 10 
min (37), it seems unlikely that this difference is due to a 
temporal requirement for uptake of CPT-cAMP. The experi-
ment in Fig. 7B shows the effect of pretreatment with CPT-
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tion. Similarly to the case for induction with NGF (Fig. 4), 
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added during the last 90 min of incubation and was partially 
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Such results again point to a difference between the actions of 
6-TG and 2-AP.

Although stimulation of ODC activity by EGF was sensitive to 
6-TG and 2-AP, it also appeared to use a pathway that could be 
distinguished from that used by NGF. In contrast to the case for NGF, 30 min of pretreatment with EGF was not 
sufficient to prevent inhibition of ODC induction by 6-TG (data not shown).

DISCUSSION

The present studies extend past work (25, 26, 38) on the 
use of purine analogues to dissect the NGF mechanism of 
action. In particular, experiments were focused on the induc-
tion of ODC. Induction of this enzyme by NGF is rapid and 
involve large increases in transcription of the ODC gene (24), 
in ODC mRNA levels (20), and in ODC enzymatic activity 
(18, 19, 22).

We show here that the purine analogues 6-TG and 2-AP 
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One potential issue is that the effects of the analogues on ODC induction could be due to nonspecific alteration of cellular metabolism. In particular, the analogues can affect cellular purine metabolism and lead, over a time course of several hours, to decreases in intracellular levels of ATP and GTP (39). The strongest counter to such a possibility is the observation that 6-TG was effective in suppressing ODC induction only if present during the first 3 min of NGF exposure. Thus, even when this analogue was present for the final 297 min of the 300-min treatment, it did not affect ODC induction. This strongly appears to rule out nonspecific effects of the analogues on cellular metabolism. The inability of 6-TG to block NGF-promoted neuronal survival, rapid protein phosphorylations, or induction of c-fos (25) again argue against general nonspecific effects of this drug with respect to inhibition of ODC induction. Similarly, the capacity of 2-AP to block a rapid response such as c-fos induction, the ability of this drug to suppress ODC induction even during the last hour of incubation, and the inability of 2-AP to inhibit NGF-promoted phosphorylations all argue against its actions being due to depletion of intracellular ATP or GTP. Work from other laboratories also supports a specificity in the actions of 2-AP. For instance, it has been reported that 2-AP blocks gene induction by interferons, but not by heat shock (45).

By what means might purine analogues affect responses to NGF, including ODC induction? In particular, what type of mechanism could account for the capacity of purine analogues to block some responses to NGF and not others and for 6-TG to suppress ODC induction only if administered during the first 3 min of NGF exposure? One possibility is by interfering with a component of a rapid signal transduction mechanism such as protein kinase activity. Purine analogues have been shown to effectively block certain kinase activities in vitro (40). In particular, 2-AP, although by no means a general inhibitor of protein kinases, can suppress several specific phosphorylation activities (40, 41, 45). It has been suggested that interference with protein kinase activity could be responsible for the observed capacity of 2-AP to block growth factor-dependent induction of β-interferon, c-fos and c-myc mRNAs (42).

In a recent study (25), we explored the inhibition of PC12 cell protein kinase activities by 6-TG and 2-AP. Both agents blocked the in vitro activity of a semi-purified NGF-stimulated kinase designated protein kinase N. Protein kinase N is an apparently novel serine kinase that is activated in PC12 cells within several minutes of NGF exposure (27, 37, 43). The dose-response relationships for in vitro inhibition of protein kinase N by purine analogues were similar to those for in vivo inhibition of NGF-promoted ODC induction and neurite regeneration. In consonance with past studies, 2-AP appeared to block PC12 cell kinase activities in addition to protein kinase N. By contrast, a variety of approaches failed to uncover other protein kinase activities in addition to protein kinase N that are sensitive to 6-TG. Such findings led to the hypothesis that protein kinase N may be required in certain pathways of the NGF mechanism and that interference by purine analogues with NGF responses such as ODC induction could be due to inhibition of protein kinase N. It was further suggested that the broader range of 2-AP as a kinase inhibitor accounts for its capacity to block NGF responses in addition to those affected by 6-TG.

The present findings are consistent with the above model. The 3-min window during which ODC induction is vulnerable to blockade by 6-TG corresponds also to the time required for protein kinase N activation by NGF (27, 38). The blockade of ODC induction by 2-AP, even when added relatively late after NGF, could well stem from the inhibition of additional protein kinase activities.

As in other cell types, permeant cAMP analogues induce ODC in PC12 cells (22). Experiments with PC12 cell variants defective in cAMP-dependent protein kinase activity have demonstrated that the latter is required for ODC induction by cAMP, but not NGF (23). However, despite this difference in primary pathways of action of NGF and cAMP analogues, 6-TG and 2-AP both inhibited cAMP-mediated induction of ODC and did so at concentrations comparable to those at which they block responses to NGF. Of relevance, past studies indicate that 6-TG and 2-AP have no apparent effect on cAMP-dependent protein kinase activity (25). On the other hand, we found here that in contrast to observations with NGF, if addition of 6-TG was delayed for as much as 1–2 h following addition of the permeant cAMP analogue, there was still inhibition of ODC induction.

The above points indicate that there are both similarities and divergences in the mechanisms by which cAMP and NGF induce ODC. These findings can be interpreted with respect to protein kinase N. The cAMP derivatives have been shown to lead to activation of protein kinase N in PC12 cells (27, 37, 43) and to do so via a mechanism dependent on cAMP-dependent protein kinase (37). Thus, it is conceivable that secondary protein kinase N activation is required for ODC induction by cAMP and that the purine analogues interfere with cAMP-promoted induction of ODC by virtue of their inhibition of protein kinase N. The differences in time of susceptibility to 6-TG could indicate that protein kinase N plays a role at different temporal stages in the mechanisms by which cAMP and NGF induce ODC.

Our observations with EGF raise similar considerations. EGF and NGF utilize different primary pathways for induction of ODC (44). Nevertheless, EGF-dependent ODC induction was sensitive to purine analogues. EGF also activates PC12 cell protein kinase N activity (27, 37). This is consistent with a possible role for protein kinase N in EGF-promoted ODC induction. However, the finding that 6-TG can block ODC activation even after 30 min of EGF pretreatment indicates, as do cAMP derivatives, that PKN would have to be required at a relatively later step in the EGF mechanism.

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