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Deprivation of nerve growth factor rapidly increases purine efflux from cultured sympathetic neurons

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The efflux of [^{3}H]purines from cultured sympathetic neurons prelabelled with [^{3}H]adenine is accelerated 2–3-fold within hours of nerve growth factor (NGF) withdrawal and is reduced by readdition of NGF. Addition of 8-(4-chlorphenyl-thio) cAMP, which delays neurite degeneration, reduced the enhanced efflux of purines, as did the addition of cycloheximide, $MgCl_2$ and the protease inhibitor tosyl-L-lysine chloromethyl ketone. Colchicine accelerated purine efflux and neurite degeneration but 2-deoxyglucose increased purine efflux without inducing degeneration, suggesting that ATP reduction itself is not the cause of neurite degeneration. The increase in purine efflux is thus an early biochemical event that has diagnostic value for the study of NGF action since deprivation is detected well before irreversible changes become established.

Nerve growth factor; Sympathetic neuron; cyclic AMP; ATP

1. INTRODUCTION

Nerve growth factor (NGF) is a neurotrophic factor that supports the survival of sympathetic neurons in vivo [1,2] and in vitro and promotes neurite outgrowth from normal and transformed neural crest derived neurons [3] in vitro. The mechanism of NGF action is only partially characterised (for reviews, see [4–6]). When NGF is withdrawn from neurite-bearing sympathetic neurons in culture, a sequence of degenerative events occurs, first evident by changes in neurite morphology and followed by the degradation and death of the cell body. Inhibitors of protein and RNA synthesis have been shown to delay degeneration for 2–3 days if added within 12 h of NGF withdrawal [7], implying that specific proteins actively promote degeneration. Degeneration may also be blocked for several days with cyclic AMP analogues following NGF withdrawal [8]. To begin an analysis of the biochemistry of neurite degradation, we looked for a simple, noninvasive assay that would indicate the absence of NGF before overt signs of degeneration are apparent. We have shown previously [9] that in sympathetic neurons the amount of [^{3}H]ATP produced metabolically from [^{3}H]adenine is proportional to neurite outgrowth and neurite ATP concentrations change in response to metabolic pressures and demands. A decrease in ATP is compensated by increases in AMP/IMP, causing the generation and efflux of labelled purine nucleosides and bases into the medium. Since ATP is crucial for axonal transport and maintenance of neurite functions [10–12], we decided to test whether changes in ATP concentration occurred as a result of NGF withdrawal and if so, whether the release of labelled purines could be developed into an assay of NGF deprivation.

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Abbreviations: NGF, nerve growth factor; CPTcAMP, 8-(4-chlorphenyl-thio)cAMP; COL, colchicine; CH, cycloheximide; TLCK, tosyl-L-lysine chloromethyl ketone

2. MATERIALS AND METHODS

Sympathetic neurons were isolated from superior cervical ganglia of 1 day postnatal rats by incubating the desheathed

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ganglia in a solution of 0.1% trypsin (Difco 1:250) containing 0.4 mM EDTA, 140 mM NaCl, 3.5 mM KCl, 10 mM phosphate buffer at pH 7.4 and 1 μg/ml DNase I (Sigma) for 30 min at 37°C. The ganglia were washed in modified L15 (Gibco)-air medium [13] and triturated 10–20 times up and down in a flame-polished pasteur pipette of not less than 1 mm aperture. Cells were spun (setting 2 for 3 min, Denley microfuge), resuspended in ~30 μl plating medium and plated in 1 μl drops onto the center of 12 mm coverslips that had been coated with 0.15 OD collagen (A_{280}) [14]. Cells were grown in L15-CO_2 [13] containing 20 μM cytosine arabinoside, 10 μM each of uridine and fluorodeoxy-uridine, 3% rat serum and 20 ng/ml 2.5 S NGF. Although ARA-C has been shown to inhibit the survival-promoting actions of NGF on sensory and ciliary ganglion neurons [15], its presence in the growth medium did not affect neuronal survival, probably because there is sufficiently high levels of 2-deoxycytidine in the rat serum.

Cells were labelled with [^{3}H]Ade [9] for 15–24 h. Samples of medium (20 μl) were removed to assess total uptake, coverslips were blotted onto tissue [9] and washed in bacteriological grade dishes (Sterilin, UK) 3 times in 10 ml L15-air medium heated at 37°C, 10 min per wash. The Sterilin plastic bound and effectively depleted all the dissociable NGF and growth medium preincubated in Sterilin dishes was unable to support neurite outgrowth on freshly derived neurons or explants. Degeneration of single cell and explant cultures after incubation in Sterilin dishes was similar to those obtained with anti-2.4 S NGF antibody (Amersham International plc) used at concentrations that block NGF action. To measure purine efflux, coverslips were put into wells containing 400–500 μl medium and 25 μl were removed at various times into 1 ml of 0.1 N HCl. At the end of the experiment, coverslips were washed briefly in L15-air and the remaining radioactivity in the cells was extracted in 1 ml of 0.1 N HCl. Radioactivity was measured by adding 3.5 ml scintillant (Beckman MS). The distribution of label in nucleotides, nucleosides and bases was measured as described previously [9]. 8-(4-Chlorphenyl-thio) cAMP (CPTcAMP) was obtained from Boehringer Corporation. Colchicine (COL), 2-deoxyglucose (dGlc), cycloheximide (CH) and tosyl-L-lysine chloromethyl ketone (TLCK) were from Sigma Chemical Co.

3. RESULTS

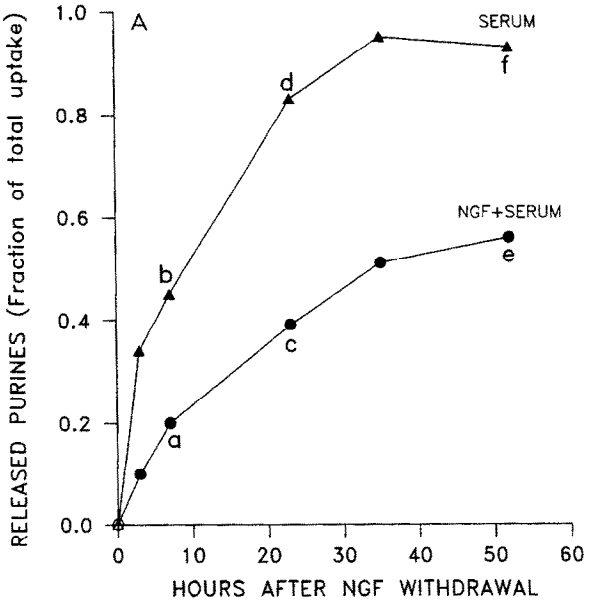
3.1. *Purine efflux from NGF-deprived neurons*

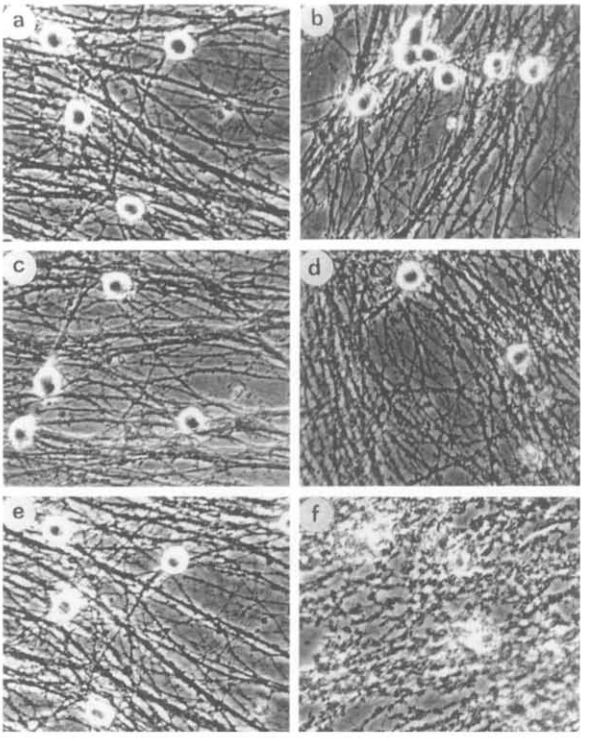
A time course of release of [^{3}H]purines from neurons grown for 1 week in medium containing 20 ng/ml NGF and 3% rat serum (full medium), preincubated with 10 μCi/ml [^{3}H]Ade (0.38 μM) and then transferred to NGF-free medium (containing 3% rat serum) is shown in fig.1A. The neurons are shown in the adjacent photograph (at 7, 26 and 52 h). A significant rise in the rate of purine efflux was observed within 3–7 h of NGF removal with no apparent morphological change in the appearance of the cells. At 26 h there was only slight degeneration of neurites yet almost all of the label was released. At this time, addition of NGF rescued over 80% of the cells (not shown). By 52 h, neurites had assumed a typical granulated appearance and neurons could no longer be rescued by NGF. Average values of fractional purine release sampled at 6–7 and 24–26 h were 17.1 ± 3 and 40.3 ± 8.7 (full medium) and 37 ± 7.9 and 82 ± 13 (NGF-free medium), respectively (3 experiments ± SD). Similar kinetics of purine release in response to NGF deprivation were obtained when explants were used instead of single cell cultures (data not shown).

3.2. *Validation of the diagnostic value of the purine efflux assay using CPTcAMP*

Survival of sympathetic and sensory neurons can also be maintained by cyclic AMP analogues, which appear to act via a different mechanism to that of NGF [8]. We therefore tested whether purine efflux would remain enhanced in the presence of CPTcAMP. As shown in fig.1B, the rate of efflux of labelled purines was reduced by 60–80% by adding 0.5 mM CPTcAMP to the NGF-free medium and the degeneration of the neurites growing in serum was also substantially retarded in the presence of CPTcAMP, as reported previously [8]. Thus, the suppression of enhanced purine efflux was not dependent on whether NGF or CPTcAMP were the survival-promoting factors. In both cases, the rate of early purine efflux

Fig.1. Efflux of [^{3}H]purines from neurons after withdrawal of NGF. (A) Sympathetic neurons (7 days in culture) were labelled for 20 h, washed and transferred to 500 μl NGF-free or full medium containing 384 ng/ml 2.5 S NGF. The amount of purines released as a fraction of total radioactivity incorporated into the cells at time zero is shown on the ordinate. Coverslips carried an average of 700–1000 neurons and 40–100 non-neural cells. At the end of the experiments non-neural cells incorporated an average of 119 ± 72 fmol/coverslip (n = 7, SD), compared to the neurons which incorporated 4869 ± 678 fmol/coverslip (n = 7, SD). Initial uptake was 8457 ± 1029 fmol/coverslip (n = 7, SD). The right hand panel shows photomicrographs of the neurons taken after 7, 26 and 52 h (magnification × 1480). (B) The experiment was conducted in parallel to that described in A. CPTcAMP (0.5 mM) was added to the withdrawal media. At the end of the experiment neurons in FM contained 2264 fmol/coverslip while those in serum contained 1836 fmol/coverslip. Photomicrographs were taken after 7, 26 and 52 h (magnification × 1450).


A
SERUM
NGF+SERUM
RELEASED PURINES (Fraction of total uptake)
HOURS AFTER NGF WITHDRAWAL
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
a
b
c
d
e
f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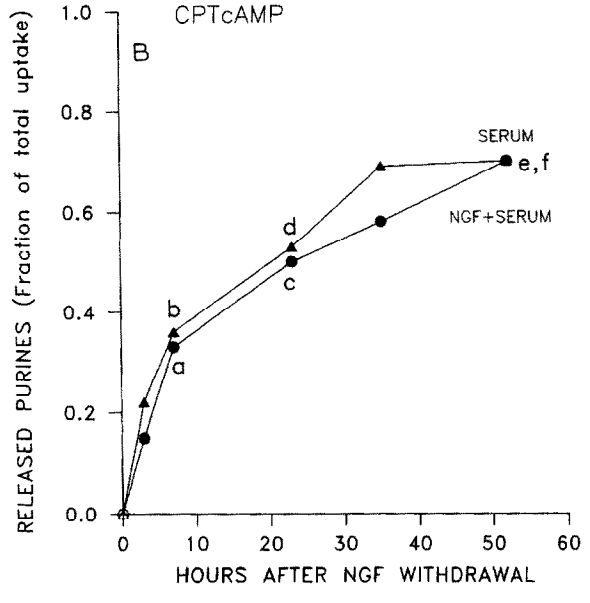
CPTcAMP
B
SERUM
NGF+SERUM
e,f
RELEASED PURINES (Fraction of total uptake)
HOURS AFTER NGF WITHDRAWAL
1.0
0.8
0.6
0.4
0.2
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10
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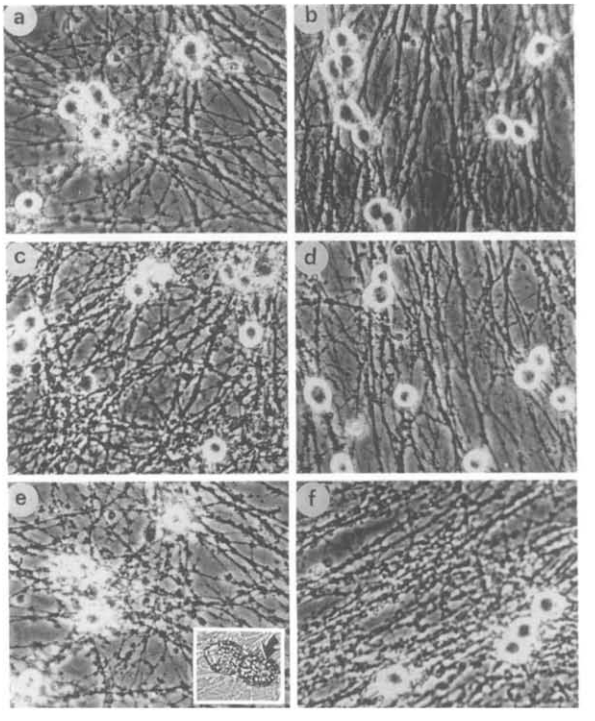
a
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and the subsequent degree of degradation were closely correlated, although no visible signs of neurite degeneration were apparent during the initial phases of accelerated purine efflux. However, although CPTcAMP could replace NGF and suppress enhanced purine efflux when added 12 h after the NGF withdrawal, we were unable to obtain any effect of CPTcAMP when it was added to newly plated cultures. This result differs from that of Rydel and Greene [8] and may reflect the much lower concentration of serum used in our experiments (3% rat serum) compared to their experiments (10% horse serum and 5% fetal calf serum).

When CPTcAMP was added to full medium, ~15% more label was released compared to the full medium control and in some instances lipid inclusion bodies accumulated in the cell bodies (fig.1B, inset in panel e). None ever appeared in cultures in the presence of CPTcAMP in NGF-free medium. This finding was not, however, reproducible in every culture and the reason for this is unclear.

3.3. *ATP degradation is not the primary cause of neurite degeneration*

The release of the Ado and Ino per se was not the cause of neurite degradation as their release could be blocked by *p*-(nitrobenzyl) thioinosine [9,16] with no change in the kinetics of release or cellular viability. The only apparent effect of the nucleoside-transport blocker was that almost all of the label appeared in hypoxanthine, as expected [9]. Since it was shown [9] that between 2/3 and 3/4 of metabolically labelled ATP from Ade is localised in neurites when they are ~5–6 mm long (after ~1 week) [9], and considering that ATP utilisation is vital for neurite function [10–12], the question arose whether the loss of ATP from the neurites was the primary cause of neurite degeneration. To examine this question neurons were exposed to dGlc which indirectly generates AMP/IMP from ATP and consequently enhances purine efflux [9]. Although 20 mM dGlc in full medium caused a decrease in steady-state ATP of 5–15%, and accelerated purine efflux, there was no effect on neurite integrity during the time neurites normally degraded in NGF-free medium. Thus, the average fraction of label remaining in the neurons 19 h after transfer of 4 day cultures into NGF-free medium or full medium supplemented with dGlc was 18.8% and 14.3% compared to 39.3% in NGF-free medium containing CPTcAMP (triplicates had less than 10% error), yet only in the NGF-free medium did the neurites become degraded. These results suggest that a decrease of 5–15% in ATP is insufficient to cause neurite degeneration and the breakdown of ATP is secondary to some other process.

3.4. *Other effectors of purine efflux*

To extend the correlation between enhanced purine efflux and subsequent degeneration and to study what may cause ATP degradation we exposed neurons to CH, which delays neuronal death after removal of NGF [7] and should thus delay purine efflux. Neurons were also exposed to 20 mM $MgCl_2$ which can block calcium entry in sympathetic neurons [17,18] and to TLCK to inhibit proteases, as suggested by Baker [19]. The effect of COL, which accelerates neurite degeneration, was also examined. The addition of CH blocked the purine release in the absence of NGF, and only slight neurite degradation was apparent up to 48 h (8 independent experiments). $MgCl_2$ and TLCK both delayed purine release by about 10 h (2 experiments) and prolonged the life time of the neurites in NGF-free medium by about 12–24 h after which the rate of purine efflux rose, assuming the enhanced rate observed in NGF-free medium and neurites degraded irreversibly. On the other hand, COL accelerated purine release by 2–3-fold (4 experiments), nearly doubling the rate of neurite degradation in the presence of NGF. The effects of NGF deprivation and COL on purine efflux were additive. The results are summarised in fig.2 which shows the fold increase in purine efflux at 10 h compared to that found without any additions to the medium with NGF and is related to the presence or absence of neurite degeneration at 24 h.

The possibility that ARA-C, normally present in our medium, may enhance purine efflux in the absence of NGF was also tested. After 11 h in NGF-free medium, efflux increased 1.63 ± 0.06-fold in the absence of ARA-C and 1.82 ± 0.08-fold in the presence of ARA-C (n = 3, SD) with no apparent change in cellular morphology compared to cultures containing NGF. This suggests that ARA-C has no effect on purine efflux,

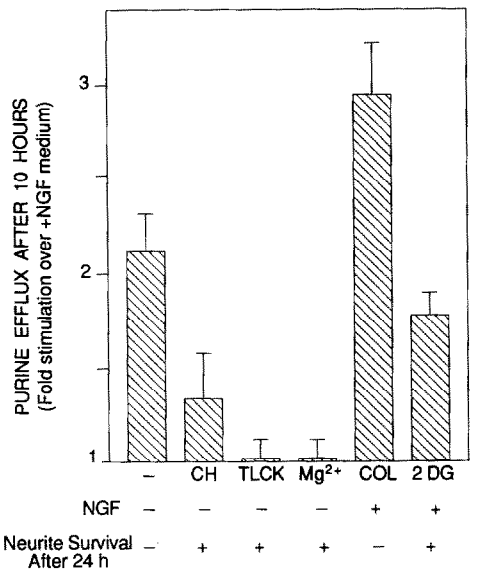

Fig.2. Effect of different additives on purine efflux and neurite degeneration. The fold efflux of purines after 10 h exposure to the different conditions is shown compared to that found without additions in medium plus NGF. The presence or absence of neurite degeneration at 24 h is also indicated.

probably due to the rat serum containing sufficient 2-deoxycytidine to inhibit any effects of ARA-C [15].

4. DISCUSSION

The acceleration of purine efflux from established, growing neurons is one of the earliest biochemical changes that have been reported to occur as a result of NGF withdrawal. The enhanced purine efflux precedes visible degenerative morphological changes and can be measured several hours before the reported appearance of adenylate kinase activity in the medium [7]. Measurement of purine efflux can thus provide the basis for a noninvasive assay which tests the ability of factors to promote the survival of NGF-dependent neurons in the absence of NGF. This conclusion is supported by the observation that enhanced purine efflux can be arrested by re-adding NGF, and that CPTcAMP and CH, which prevent neurite degeneration [7,8], retard purine efflux in the absence of NGF.

There are clearly multiple pathways promoting neuronal survival, as has been shown with cAMP analogues which act via different mechanisms to NGF in primary neurons [8], in chromaffin cells [20] or in PC12 cells [21–24]. CPTcAMP may be acting in conjunction with serum factors to stabilise NGF-induced proteins localised in the neurites. Such an action would explain why the efficacy of CPTcAMP in prolonging the life of neurons deprived of NGF increases with the age of the cultures, along with the enhanced capacity for survival of older neurons bearing longer neurites (results not shown). This protein could be the NGF receptor, or, perhaps, a cytoskeletal-associated protein [23]. The amount of CPTcAMP in the cells, however, is much higher than the amounts of cAMP that can accumulate upon stimulation of adenylate cyclase in the absence of drugs that block phosphodiesterase activity (Suidan and Tolkovsky, unpublished). Thus the physiological relevance of this action of cAMP action remains to be explored.

An important question is whether the reduction in ATP and the consequent purine efflux as a result of NGF withdrawal is directly relevant to the mechanism of neurite degeneration. The observation that the rate of purine efflux can be uncoupled from the rate of neurite degradation with dGlc suggests that a reduction in ATP does not necessarily promote neurite degeneration. However, the ability of $MgCl_2$ and TLCK to delay the rise in purine efflux and neurite degradation caused by the absence of NGF, but not that caused by the presence of dGlc, suggests that the ATP breakdown induced by NGF withdrawal may have some physiological significance. It has been shown that ATP gradients within cells can change coordinately with cytoskeletal rearrangement [25,26] and that calcium causes changes in cytoskeletal elements [27–29]. The results obtained with $MgCl_2$ and TLCK suggesting indirectly that there may be a slow rise in calcium as a result of NGF withdrawal are being examined in detail.

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