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Caffeine promotes survival of cultured sympathetic neurons deprived of nerve growth factor through a cAMP-dependent mechanism

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The effects of caffeine on neuronal survival independent of trophic factor support were examined in developing superior cervical ganglion in vitro. We found that caffeine promoted neuronal survival in the absence of nerve growth-factor (NGF) in a dose-dependent manner ($EC_{50} = 6$ mM). Pulse treatment with caffeine or high K^+ (40 mM), which caused only a transient increase in intracellular free Ca^{2+} levels ($[Ca^{2+}]_i$), did not promote survival. In contrast, caffeine potentiated the saving effect of various phosphodiesterase inhibitors including theophylline ($EC_{50} = 3$ mM) and 3-isobutyl-1-metylxanthine ($EC_{50} = 0.4$ mM). Non-xanthine phosphodiesterase inhibitor Ro 20–1724 potentiated the survival promoting effect of caffeine or IBMX. Indeed, administration of 20 mM caffeine rapidly restored the cAMP level of NGF-deprived neurons to normal (0.34 pmol/well) within 10 min; the level reached a plateau level (0.69 pmol/well) at 10 h. Even after 1 day, the sustained level was maintained in the presence of caffeine. In contrast, noradrenaline and isoproterenol, which cause only a transient increase in cAMP levels, did not support survival. These data, in conjunction with others, suggest that sustained levels of second messengers, including not only the $[Ca^{2+}]_i$ but also the cAMP level, would support the survival of superior cervical ganglion cells independent of trophic factor support.

Introduction

There is growing evidence that electrical activity plays a role in determining neuronal survival during development. Afferent neural input generally promotes neuronal survival. Chronic depolarization with elevated K^+ has proved to support neuronal survival in a variety of cell types in culture, including sympathetic neurons [1]. Conversely, removal of afferent input increases naturally-occurring neuronal death of ciliary ganglia [2]; the blockade of ganglionic transmission promotes neuronal death in retinal ganglion cells [3], and also in chicken sympathetic and ciliary ganglion cells [4]. However, it appears that electrical activity per se does not have a decisive role, rather the generation of second messengers or release of putative growth factors, most of them yet to be defined, is involved in this phenomenon. Recently, pharmacological evidence has been provided in chick ciliary [5], rat sympathetic [6] and cerebellar granule neurons [7], suggesting that Ca^{2+} influx through dihydropyridine-sensitive L-type channels is required for neuronal survival promoted by elevated K^+. Indeed, measurements of intracellular free Ca^{2+} levels ($[Ca^{2+}]_i$) of neurons loaded with fura-2 as a probe for monitoring Ca^{2+} have revealed that cell survival is correlated with the level of $[Ca^{2+}]_i$ of sympathetic neurons chronically exposed to various concentrations of extracellular K^+; it shows that 50% survival occurs at about 184 nM $[Ca^{2+}]_i$ and complete survival, independent of trophic support, occurs at about 240 nM $[Ca^{2+}]_i$ [8]. This and other findings [9] suggest that sustained levels of $[Ca^{2+}]_i$ appear to be pertinent to the survival-promoting effect of high K^+. However, it has not yet been critically tested whether or not a transient increase in $[Ca^{2+}]_i$, resulting from release of Ca^{2+} in internal store sites, is sufficient for neuronal survival.

cAMP has been implicated as a physiologically relevant modulator of neuronal survival; cAMP analogs prevent the death of nerve growth-factor (NGF)-deprived sympathetic neurons [10–12]. Moreover, it is well documented that presynaptic stimulation causes up-regulation of cAMP levels of superior cervical ganglia [13,14]. Rydel and Greene [12] have suggested that

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cAMP and NGF promote the survival of sympathetic neurons in vitro through distinct mechanisms. We have here examined the effects of caffeine which is known to induce a rapid and transient release of Ca^{2+} from internal store sites of sympathetic neurons [15,16]. Caffeine is also known to elevate cAMP levels by inhibiting the phosphodiesterase activity in sympathetic neurons [17]. Thus, methylxanthine derivatives may serve to perturb the intracellular second-messenger levels involved in cellular pathways supporting neuronal survival. We have found that caffeine promotes the survival of NGF-deprived sympathetic neurons, not through a 'transient' increase in $[Ca^{2+}]_i$ released from intracellular store sites, but through increasing accumulation of cAMP levels. This also supports our Ca^{2+} set-point hypothesis in which only 'sustained' levels of $[Ca^{2+}]_i$ are pertinent to neuronal survival.

Materials and Methods

Cell culture. Dissociated sympathetic neurons were prepared from superior cervical ganglia of Wistar rats (P1–2) according to the method of Johnson and Argiro [18]. Briefly, neurons were plated on collagen-coated 48-well plastic dishes (approx. 13000 cells/well) for adenylate kinase (AK) assay, or 12-well dishes (approx. 31000 cells/well) for cAMP assay. Collagen-coated glass coverslips (25 mm diameter, 0.17 mm thickness) were used for fluorescence measurements with fura-2. Neurons were fed twice a week with Eagle's minimum essential medium (MEM, Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal calf serum (Filtron Pty., Brooklyn, Australia) and 50 ng/ml NGF, and grown at 36°C in a humidified atmosphere of 5% CO_2/ 95% air. The anti-mitotic drug, fluorodeoxyuridine (20 μM) was added together with 20 μM uridine to the feeding medium for 5 days to kill non-neuronal cells. Non-neuronal cells contaminated under standard culture conditions were 10–15%. When necessary, neurons were further purified by the differential adhesion method of McCarthy and Partlow [19], and these purified neurons were treated with 20 μM fluorodeoxyuridine as well. This purification procedure reduced contamination of non-neuronal cells to less than 5%.

Evaluation of neuronal survival after NGF deprivation. Neurons were stained with 0.4% trypan blue in Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline (PBS; pH 7.2), and viable cells were counted under a phase-contrast microscope. As a quantitative measure of neuronal death, the amount of a cytoplasmic enzyme, adenylate kinase (AK), released into medium was assayed as described by Martin et al. [20]. Briefly, assay medium (0.2 ml/well) consisting of 10% fetal bovine serum, 90% MEM, was supplemented with 50 ng/ml NGF (for control) or with 0.5% antiserum against NGF (for NGF deprivation). The serum used for this assay was heat-inactivated and dialyzed against MEM for 3 days. After incubation in the assay medium for 36 h, the neurons were inspected, and the medium was taken for AK assay. The remaining cells which were attached to the collagen were solubilized with 0.2 ml of Triton X-100 in 10 mM Hepes buffer containing 200 mM NaCl (pH 7.0) and were taken for AK assay as well. A 15–20-μl aliquot of the sample was added to 0.4 ml of the assay reagent consisting of 50 mM imidazole-HCl buffer (pH 7.0) containing 1 mM ADP, 1 mM glucose, 2 mM $MgCl_2$, 100 μM NADP, 0.02% bovine serum albumin (BSA), 2 μg/ml glucose-6-phosphate dehydrogenase (Boehringer Mannheim, Germany) and 10 μg/ml yeast hexokinase (Boehringer Mannheim). The reaction was linear with regard to incubation time up to 90 min. The reaction mixture was usually incubated for 50 min at room temperature, and AK activities were calculated by measuring florescence intensities at 460 nm (slit width 4 nm) with the excitation wavelength of 340 nm (4 nm) using Shimadzu fluorescence spectrophotometer RF 502. Corrections were made for the AK content of the assay medium from parallel collagen-coated plates without cells and the 0.1% Triton solution. The AK activity of the assay medium was expressed as a percentage of the sum of the released and Triton-extracted activity. Normally, 3–5% of total AK activity was released from cultures of healthy neurons; this value increased to 30–45% upon cell death after the neurons were deprived of NGF by adding antiserum against NGF (0.5%) for 36 h and harvested for the assay. The values of released AK (%) were shown as mean ± S.E.

Measurements of $[Ca^{2+}]_i$ of sympathetic neurons loaded with fura-2. Sympathetic neurons were loaded with 2 μM fura-2 [21] for 1 h and subsequently incubated for 30 min in Hepes-buffered saline (pH 7.2) containing 0.5% BSA, as described in the literature [22]. Neurons in Hepes saline (pH 7.2) were maintained at 32–34°C during measurements. Fluorescence imaging of fura-2-loaded neurons was described in detail in our previous literature [8]. Fluorescence measurements were done on single neurons and small aggregates to collect fluorescence signals from individual neurons. We typically measured ratio values from 5–7 small areas of the cytoplasm of a single neuron at least 5 times per each area with an interval of 2–3 s, and then averaged these values. The values of $[Ca^{2+}]_i$ were shown as mean ± S.D.

cAMP assay. cAMP was assayed with the radioimmunoassay kits (Amersham International, Buckinghamshire, UK). cAMP fraction was extracted from control and caffeine-treated neurons according to the method of Kessler et al. [23]. Briefly, neurons were washed with PBS (pH 7.2), followed by addition of 300 μl of 6% trichloroacetic acid (TCA) to each well. The dishes were frozen and thawed 5 times to avoid con-

tamination of collagen substrate into samples by scraping. The TCA was recovered and centrifuged (2500 × *g*, for 15 min), and then the supernatants were washed 4 times with 1.5 ml of water-saturated diethyl ether. The washed supernatants were frozen and lyophilized. The assay was done according to the recommended procedure described by the manufacture. The values of cAMP level were shown as mean ± S.E.

Materials. Mouse NGF (2.5S) was isolated from submaxillary glands of male adult mouse by the method of Mobley et al. [24]. Antiserum against mouse 2.5S NGF [25] was kindly donated by Dr. E.M. Johnson, Jr. and Ms. P. Osborne (Department of Pharmacology, Washington University School of Medicine, St. Louis, MO, USA). Fura-2 acetoxymethyl ester was purchased from Molecular Probes (Eugene, OR, USA) or Dojin Pharmaceutical (Kumamoto, Japan). Ionomycin from Streptomyces conglobatus was obtained from Hoechst (Tokyo, Japan). Methylxanthine derivatives were obtained from Research Biochemical (Natick, MA, USA) or Sigma Chemical (St. Louis, MO, USA). Ro 20–1724 was purchased from Gibco-BRL (Gaithersburg, MD, USA). All other reagents were of reagent quality.

Results

Caffeine prevents the death of developing sympathetic neurons after acute withdrawal of NGF

Sympathetic neurons were dissociated from superior cervical ganglia of neonatal rats and were grown for 7 days in the presence of NGF. These phase-bright cells with extensive neurites underwent degeneration within 2 days in response to acute withdrawal of NGF [20]. As a quantitative measure of cell death, we measured AK activity released into medium [20]; the cells released 41.6 ± 1.3% (mean ± S.E., $n = 3$) of total AK activity after NGF deprivation for 36 h under our assay conditions, while spontaneous release from healthy neurons was 2.8 ± 0.4% ($n = 3$, Fig. 1). When the neurons were incubated in NGF-deprived medium containing 15 mM caffeine for 36 h, they looked healthy from visual inspection. However, released AK value was 12.3 ± 0.4% ($n = 3$) or 16.4 ± 2.8% ($n = 3$) in the presence or absence of NGF, respectively (Fig. 1). Since AK was released even in the presence of NGF, we considered a possibility that long-term exposure to caffeine may be toxic to non-neuronal cells. When the neurons were treated with antiserum against NGF (0.5%) on day 3, most of developing sympathetic neurons were eliminated and flat non-neuronal cells remained intact by day 7. Caffeine was toxic to these cells by visual inspection. Indeed, the released AK values reached 69.8 ± 3.6% ($n = 3$) in the presence of NGF and 65.6 ± 1.0% ($n = 3$) in the absence of NGF when these non-neuronal cells were treated with 15 mM caffeine for 36 h. Moreover, released AK was decreased to 3.1 ± 0.4%

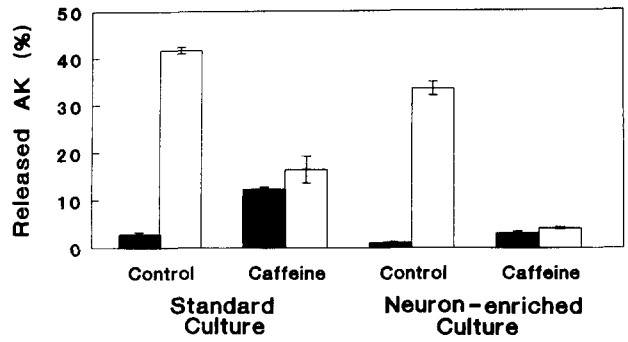

Fig. 1. Effects of caffeine on the release of adenylate kinase (AK) in sympathetic neurons under various culture conditions. Sympathetic neurons (1 week in vitro) were incubated in assay medium for 36 h in the presence of NGF (■) or in its absence (□), and then subjected to AK assay ('Standard Culture'). Caffeine (15 mM) was added to the assay medium at the same time when neurons were deprived of NGF by adding antiserum against NGF (0.5%). 'Neuron-enriched Culture' implies that sympathetic neurons were further purified as described in Materials and Methods. Values represent mean ± S.E. of three samples.

($n = 3$) in the presence of NGF and caffeine when neurons were further enriched according to the differential adhesion method described by McCarthy and Parlow [19](Fig. 1). Upon deprivation of NGF, released AK remained as 4.1 ± 0.3% ($n = 3$), indicating that caffeine supports the survival of sympathetic neurons after acute withdrawal of NGF. Alternatively, it was also possible to work with cells under standard culture conditions if caffeine was added to the assay medium at 12 h after deprivation of NGF to reduce its toxicity. No obvious deterioration of neurons was observed up to 12 h after NGF deprivation (see also Fig. 8). Under these conditions the basal level of released AK was 8.8 ± 0.1% ($n = 3$) in the presence of caffeine (15 –20 mM), and only a slight increase in released AK was observed after NGF deprivation for 36 h (13.0 ± 2.8% at 15 mM caffeine, 10.9 ± 0.4% at 20 mM caffeine, Fig. 3). Consistent with the data on released AK, photomicrographs shown in Fig. 2 support the proposal that caffeine saves NGF-deprived neurons from cell death in vitro (A,E: control cells; B,F: NGF-deprived cells; C,G: cells in the presence of NGF and caffeine; D,H: NGF-deprived cells in the presence of caffeine). The possibility that Schwann cells mediate the saving effect of caffeine is unlikely, since conditioned medium prepared by treating pure Schwann cells (prepared by the procedure developed by Manthorpe et al. [26]), with caffeine did not save the neuron from cell death (data not shown).

Fig. 3 shows the dose-response curve of the saving effect of caffeine, indicating that EC_{50} is 6 mM. These findings indicate that caffeine is toxic primarily to non-neuronal cells, and demonstrate that caffeine at ≧ 15 mM promotes the survival of developing sympathetic neurons after deprivation of NGF.

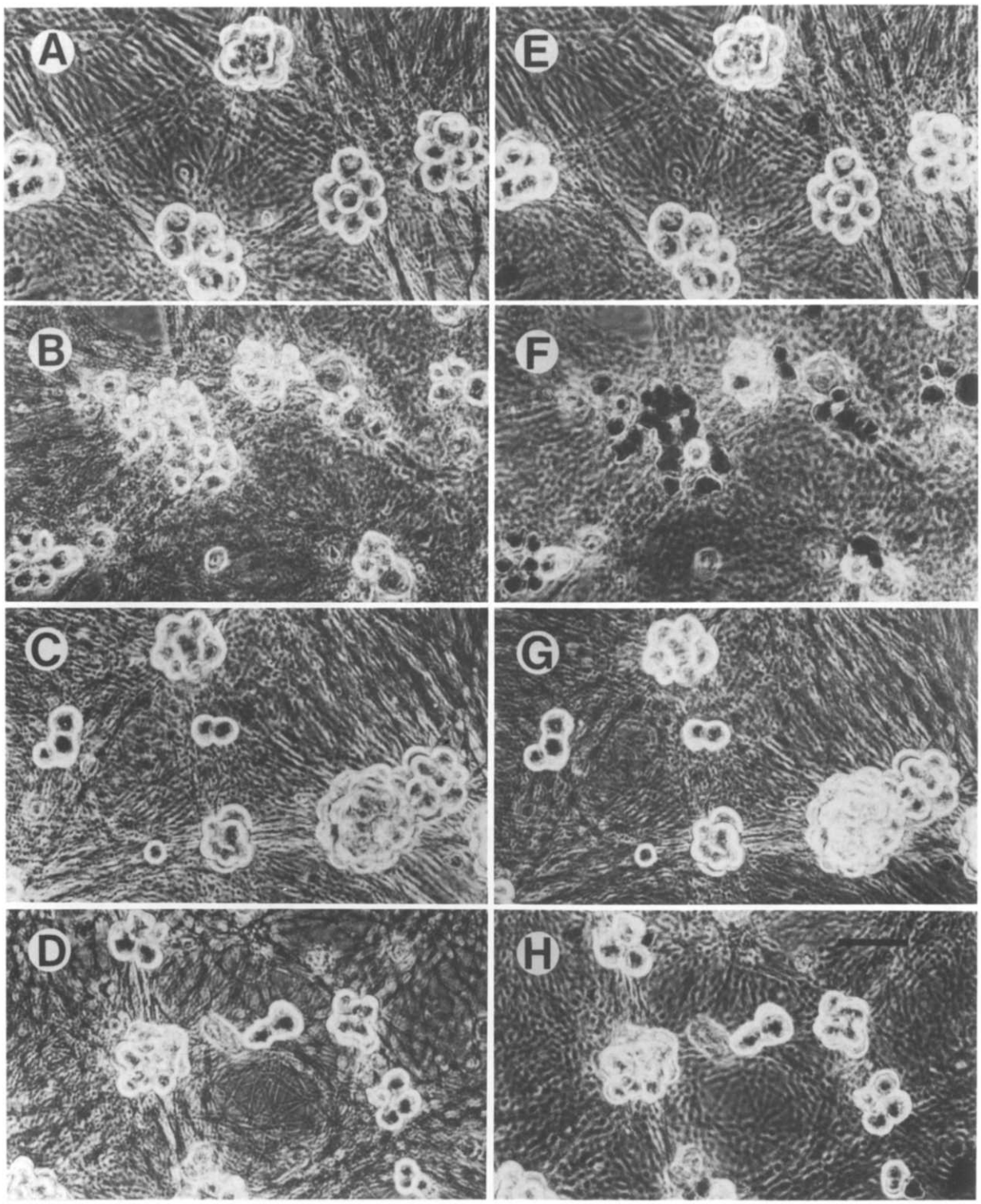

Fig. 2. Phase-contrast micrographs of sympathetic neurons after NGF deprivation. Sympathetic neurons maintained for 1 week in the presence of NGF in vitro were cultured for 36 h in the presence of NGF (A) or in its absence (B), and photographed. The neurons were exposed to 20 mM caffeine at 12 h after NGF deprivation, cultured for a further 24 h, and photographed (D). Control cultures received caffeine for 24 h in the presence of NGF (C). Photomicrographs E, F, G, or H show results obtained with trypan blue staining of the same fields corresponding to A, B, C, or D, respectively. The bar represents 100 μm.

The action of caffeine which causes a transient increase in $[Ca^{2+}]_i$ is not sufficient for sympathetic neurons to survive in the absence of NGF

Caffeine has been known to induce a rapid release of Ca^{2+} from internal stores [15,16]. This release occurs only transiently because of a limited capacity of internal storage. Thus, the basal level of $[Ca^{2+}]_i$ reverts back to normal in sympathetic neurons in the presence of this drug, unless external Ca^{2+} is supplied through the activation of voltage-sensitive Ca^{2+} channels [15,16]. We confirmed the transient nature of elevated $[Ca^{2+}]_i$ evoked by caffeine; a transient increase of $[Ca^{2+}]_i$ (237 ± 63.6 nM, mean $\pm$ S.D., $n = 15$) induced by 20 mM caffeine was diminished within 20–30 s (Fig. 4A), and there was no significant difference in the basal levels of $[Ca^{2+}]_i$ when the neurons were treated with 20 mM caffeine for 4–9.5 h. For example, the basal level of $[Ca^{2+}]_i$ of the sympathetic neurons treated with caffeine for 7 h was 87.8 ± 31.9 nM ($n = 40$), which compared well with that of untreated neurons (73.1 ± 39.4 nM, $n = 40$, in Fig. 4B Control). We then asked whether the saving effect of caffeine originate from its short-term activity to release Ca^{2+} from internal storage sites. Pulse application of caffeine for 30 min did not save neurons from cell death (data not shown). Conversely, when the neurons were exposed to caffeine (15 mM) at 30 min prior to NGF deprivation, and incubated with caffeine in the absence of NGF for a total of 36 h, the saving effect of this drug was observed; released AK value was $13.8 \pm 0.7\%$ (mean $\pm$ S.E., $n = 3$) or $16.3 \pm 0.6\%$ ($n = 3$) in the presence of NGF or in its absence, respectively. Moreover, the saving effect of caffeine was not abolished by treating the neurons with 10 μM dantrolene (data not shown), which has been proven to block the release of internal Ca^{2+} induced by caffeine [16]. Recently, Johnson and his coworkers [20] have postulated the presence of a

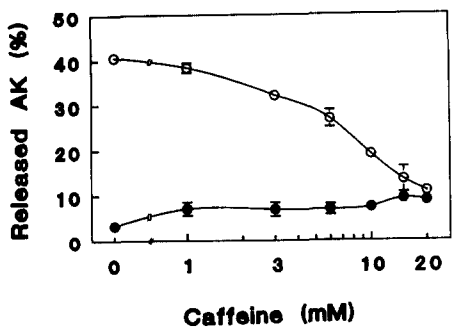

Fig. 3. Dose-response curve of caffeine for the survival of NGF-deprived sympathetic neurons in culture. Sympathetic neurons were cultured for 36 h in the presence (•) or absence (○) of NGF, and then assayed for released AK. Caffeine was added to the medium at 12 h after NGF deprivation. The EC_{50} of the saving effect of caffeine was 6 mM. Each bar represents mean $\pm$ S.E. of triplicate values. The data without S.E. imply that it was too small to be included in this figure.

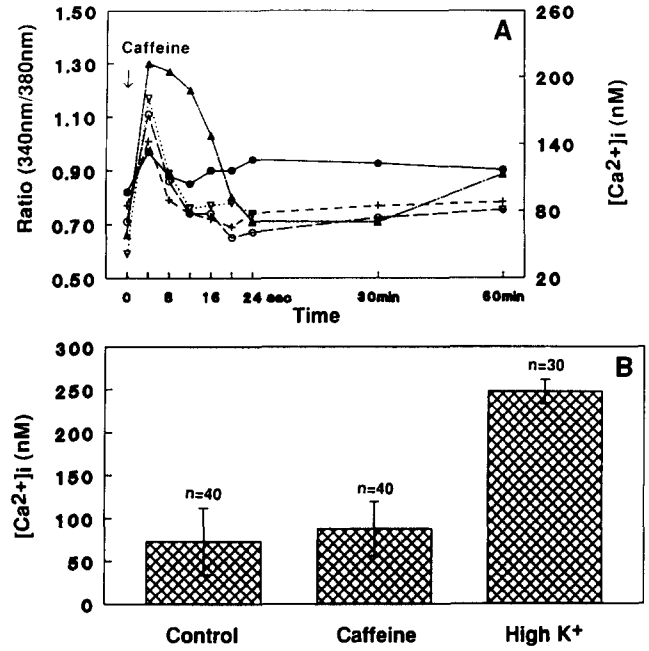

Fig. 4. Effect of caffeine on $[Ca^{2+}]_i$ levels of sympathetic neurons. A. Caffeine-induced transient increase in $[Ca^{2+}]_i$ levels of individual neurons: Sympathetic neurons were loaded with fura-2, and $[Ca^{2+}]_i$ levels of 5 individual neurons were measured at the same time as described in Materials and Methods. Neurons were exposed to 20 mM caffeine at 0 time. B. $[Ca^{2+}]_i$ levels of neurons treated chronically with caffeine or high-K^+: Neurons were treated with caffeine (20 mM, center) or high K^+ (35 mM, right) for 7 h, and then their $[Ca^{2+}]_i$ levels were measured (the number of cells is indicated). $[Ca^{2+}]_i$ levels of untreated neurons are shown as control. Mean $\pm$ S.D.

degeneration cascade leading to cell death, based on the fact that cycloheximide could no longer prevent the death of more than half of the neurons at 18 h after NGF deprivation. Thus, this raises a possibility that a transient elevation of $[Ca^{2+}]_i$ during a particular step of this cascade might be sufficient to prevent this degeneration cascade. Under our culture conditions the commitment point for the saving effect of cycloheximide occurred at 15 h (Fig. 8). Caffeine, however, did not save the neurons from cell death when the neurons were treated with caffeine for 30 min at various time points (0, 2, 10, 15, 20 h) after NGF deprivation. For comparison, we treated the neurons with high K^+ for 30 min as well. Neither of these treatments gave any saving effect (data not shown). Finally, effects of combinations of caffeine and various concentrations of K^+ were examined. We have previously reported that high K^+ prevents the death of NGF-deprived neurons through influx of Ca^{2+} via dihydropyridine-sensitive Ca^{2+} channels [6]. Caffeine at 6 mM (EC_{50}) did not potentiate the saving effect of K^+. For example, released AK values were $26.1 \pm 1.6\%$ and $24.5 \pm 2.9\%$ in the presence of 6 mM caffeine alone and 25 mM K^+ alone, respectively, and the combination of the two drugs reduced the released AK to $13.4 \pm 1.1\%$ instead

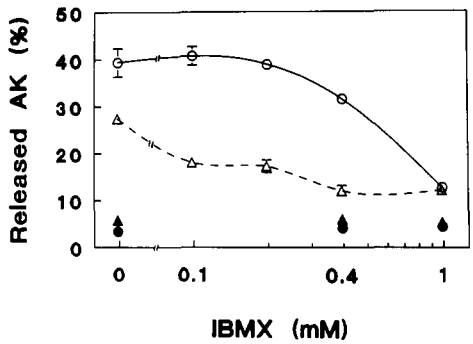

Fig. 5. Potentiation of the saving effect of 6 mM caffeine by the presence of various concentrations of IBMX. Sympathetic neurons, which had been deprived of NGF for 12 h, were exposed to various concentrations of IBMX (0.1–1 mM) for a subsequent 24 h in the presence of caffeine (6 mM) or in its absence. The neurons were then subjected to AK assay. Symbol: ●, +NGF; ○, −NGF; ▲, +NGF/+caffeine; △, −NGF/+caffeine. Mean ± S.E. ($n = 3$). The data without S.E. imply that it was too small to be included in this figure.

of 11.3 ± 2.2%, a simple sum of the two effects. Thus, it is safe to conclude that the saving effect of caffeine was not mediated through its transient activity to enhance Ca^{2+} release from internal storage sites. This conclusion further supports the notion that a sustained, but not transient, level of $[Ca^{2+}]_i$ is required for the saving effect of Ca^{2+} to manifest itself.

The saving effect of caffeine is mediated through its inhibitory activity against cAMP phosphodiesterase

The saving effect of caffeine may be mediated through increasing accumulation of cAMP by inhibiting cAMP phosphodiesterase in sympathetic neurons [17]. We have employed various methylxanthine derivatives to examine whether there is any correlation between K_i values for phosphodiesterase and the saving effects of these drugs. Fig. 5 shows 3-isobutyl-1-methylxanthine (IBMX) prevented the death of these neurons after NGF deprivation (EC_{50} is 0.4 mM), and potentiated the saving effect of 6 mM caffeine (EC_{50}). For example, released AK values were 26.1 ± 1.6% ($n = 3$) and 38.9 ± 0.1% ($n = 3$) in the presence of 6 mM caffeine alone and 0.2 mM IBMX alone, respectively. The combination of the two drugs reduced the released AK to 17.3 ± 1.3% ($n = 3$) instead of 25.7 ± 1.6%, a simple sum of the two effects (Student's t-test, $P < 0.005$). Similarly, theophylline promoted the survival of the neurons (EC_{50} is 3 mM), and also potentiated the effect of 6 mM caffeine (Fig. 6); released AK values were 25.0 ± 0.7% ($n = 3$) and 38.3 ± 0.6% ($n = 3$) in the presence of 6 mM caffeine alone and 1 mM theophylline alone, respectively, and the combination of the two drugs reduced the released AK to 17.8 ± 0.9% ($n = 3$) instead of 24.2 ± 1.2%, a simple sum of the two effects (Student's t-test, $p < 0.005$). Thus, the order of potency of the saving effects of these derivatives is IBMX (EC_{50}; 0.4 mM) > theophylline (3 mM) > caffeine (6 mM), which is exactly the order of potency as cAMP phosphodiesterase inhibitors (IBMX (EC_{50}; 10 mM) > theophylline (150 mM) > caffeine (250 mM)) [27,28]. This correlation suggests that the drugs are acting to support cell survival after NGF deprivation, in part or in whole, through their effects on cAMP levels. To exclude the possibility that caffeine act through the binding to adenosine receptors, we tried to use Ro 20–1724, a non-xanthine phosphodiesterase inhibitor which does not act as an adenosine receptor antagonist; Ro 20–1724 at the concentration of up to 100 μM [29] did not support neuronal survival after NGF deprivation (released AK values was 40.1 ± 0.7%, $n = 2$). However, the combination of 100 μM Ro 20–1724 with methylxanthine derivatives reduced the released AK to 15.4 ± 0.2% (for 6 mM caffeine, $n = 3$, Student's t-test, $P < 0.005$) and 23.5 ± 0.5% (for 0.2 mM IBMX, $n = 2$, Student's t-test, $P < 0.005$), respectively, while released AK values were 18.9 ± 0.7% ($n = 3$) and 38.9 ± 0.1% ($n = 2$) in the presence of 6 mM caffeine alone and 0.2 mM IBMX alone, respectively. These findings support our conclusion that caffeine increases cAMP levels not through adenosine receptor dependent mechanism, through its activity as a phosphodiesterase inhibitor.

To obtain direct evidence, we went on to measure cAMP levels of sympathetic neurons treated with caffeine. The cAMP level of sympathetic neurons was 0.403 ± 0.015 pmol/well (mean ± S.E., $n = 4$) which compared well to other measurements [17]. This value was decreased to 0.253 ± 0.009 pmol/well ($n = 4$, 63% of the original level) at 12 h after NGF deprivation (Fig. 7). Treatment with caffeine (20 mM) restored the

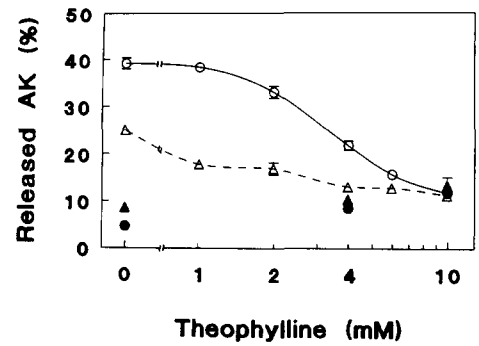

Fig. 6. Potentiation of the saving effect of 6 mM caffeine by the presence of various concentrations of theophylline. Sympathetic neurons, which had been deprived of NGF for 12 h, were incubated for a further 24 h in the presence or absence of caffeine (6 mM) in assay medium containing various concentration of theophylline (1– 10 mM). The neurons were then subjected to AK assay. Symbol: ●, +NGF; ○, −NGF; ▲, +NGF/+caffeine; △, −NGF/+caffeine. Mean ± S.E. ($n = 3$). The data without S.E. imply that it was too small to be included in this figure.

cAMP level back to normal within 10 min (0.43 ± 0.052 pmol/well, $n = 4$), and caused a gradual increase to 0.69 ± 0.072 pmol/well (273% of the level of NGF-deprived cells, $n = 4$) by 10 h. The elevation of cAMP in response to treatment with caffeine for 10 min or 10 h was diminished within 1 h upon its withdrawal. It appears that cAMP level reached a plateau level (0.69 pmol/well) at 10 h, and then slightly decreased to 0.55 ± 0.011 pmol/well ($n = 4$) after 24 h caffeine treatment. The decrease appeared to be due to slight neuronal degeneration. Chronic treatment (for 24 h) with the combination of more potent inhibitors, IBMX (1 mM) and theophylline (5 mM), also resulted in an elevated level of cAMP (0.68 ± 0.51 pmol/well, $n = 4$). Thus, these data further support our observation that the saving effect of caffeine is well correlated with an increased cAMP level as a result of its inhibitory activity against phosphodiesterases in sympathetic neurons. We have also found that noradrenaline (10–100 μM) or isoproterenol (10– 100 μM), which are known to cause only a transient increase in cAMP level of sympathetic neurons [13,14,30], failed to support the survival of NGF-deprived neurons (data not shown). This suggests that sustained levels of cAMP are required for the saving effect of methylxanthine derivatives to manifest itself.

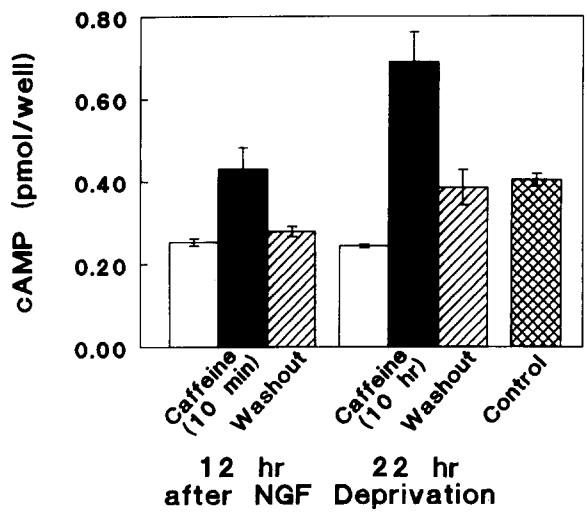

Fig. 7. Effect of caffeine on cAMP levels of sympathetic neurons deprived of NGF. Sympathetic neurons were deprived of NGF by adding antiserum against NGF (0.5%). After 12 h, neurons in some dishes were treated with 20 mM caffeine for 10 min (■, left) or 10 h (■, right; NGF deprivation for a total period of 22 h). cAMP levels of these neurons together with untreated neurons deprived of NGF for 12 h (□, left) and 22 h (□, right) were measured by using radioimmunoassay kits. Other dishes which had been received caffeine treatment for 10 min or 10 h, were washed twice with NGF-deprived medium, incubated in this medium for 1 h, and cAMP levels were then determined (Washout, ▨). A column in the extreme right shows a cAMP level of cells cultured in the presence of NGF (Control, ■). Mean ± S.E. ($n = 4$).

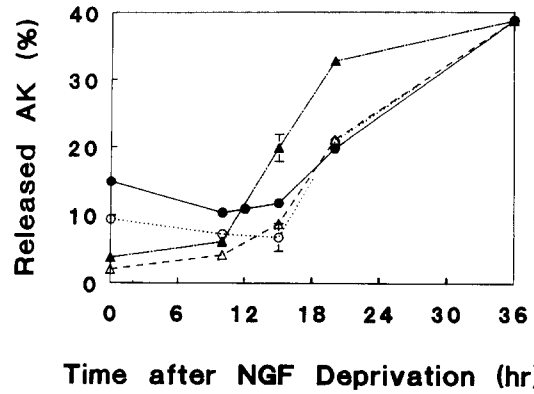

Fig. 8. Commitment point for the saving effect of methylxanthine derivatives, CPT-cAMP, or cycloheximide from neuronal death. Sympathetic neurons grown for 1 week in vitro were deprived of NGF at 0 h, and drugs were added to assay medium at the indicated time points (●, 15 mM caffeine; ○, 1 mM IBMX; △, 200 μM CPT-cAMP; ▲, 2 μM cycloheximide). The neurons were deprived of NGF for a total of 36 h, and then subjected to assay for AK. Since methylxanthine derivatives were toxic to non-neuronal cells as described in the text, released AK values from cells treated with these drugs for 36 h are higher than those treated for 21–26 h. The commitment point obtained from this figure is: 15 h for cycloheximide, 20 h for IBMX, caffeine, and CPT-cAMP. Mean ± S.E. ($n = 3$). The data without S.E. imply that it was too small to be included in this figure.

The commitment point of caffeine, IBMX, and CPT-cAMP for preventing neuronal death

Martin et al. [20] have examined the time course of the saving effect from neuronal death of cycloheximide, and determined the time point when neurons commit to die despite inhibition of protein synthesis. Thus, the commitment point is defined as a time when addition of cycloheximide to the culture medium could prevent half of the neurons from cell death. Here this point occurred at 15 h after withdrawal of NGF under our culture conditions (Fig. 8), which is close to the point reported by Martin et al. (18 h). Similarly, we determined the commitment point of caffeine (15 mM), IBMX (1 mM), and CPT-cAMP (0.2 mM), all giving the same point at 20 h, which occurs significantly later than that of cycloheximide (2 μM, Fig. 8). This suggests that cAMP prevents neuronal death at some point down stream of the cycloheximide-sensitive step in the death cascade. To confirm this, the following switchover experiments were done. NGF-deprived neurons were first incubated in the presence of 2 μM cycloheximide for 20 h, then switched over to 0.2 mM CPT-cAMP and incubated for a further 30 h; these neurons survived in the presence of cAMP (released AK; 3.5 ± 0.16%, mean ± S.E., $n = 3$). In contrast, NGF-deprived neurons were treated with 0.2 mM CPT-cAMP for 20 h and then switched over to 2 μM cycloheximide for 30 h; these neurons died even in the presence of cycloheximide (released AK; 24.5 ± 1.19%, $n = 3$). This supports the possibility that caffeine and

cAMP promote neuronal survival by acting through post-translational modification(s).

Discussion

We have provided here evidence that methylxanthine derivatives promote the survival of developing sympathetic neurons after acute deprivation of NGF. The major biological activities of these drugs are mediated through (1) translocation of intracellular Ca^{2+}, (2) an increase in cAMP levels by inhibiting of cAMP phosphodiesterase. With regard to the former possibility, we have proposed a Ca^{2+} set-point hypothesis for neuronal survival [6]. This hypothesis conjectures that developing neurons whose levels of $[Ca^{2+}]_i$ are low, would require trophic factor for survival, and neurons at elevated levels of $[Ca^{2+}]_i$ will survive autonomously, independent of trophic factor support for survival. As a consequence, when neurons have matured [30], and thus have higher levels of $[Ca^{2+}]_i$ [8], these neurons will become less dependent on trophic factor. We have provided evidence for this in sympathetic neurons [8]. This hypothesis was further tested by using caffeine and methylxanthine derivatives. Elevated levels of $[Ca^{2+}]_i$ (238 ± 64 nM) induced by caffeine (20 mM) were similar to those (247 ± 14 nM) obtained with high K^+ medium (35 mM) [8], but duration of the elevated levels was terminated within 20–30 s. We found that pulse application of either caffeine or high K^+ did not prevent neuronal death, supporting the notion that sustained levels of $[Ca^{2+}]_i$ are required for neuronal survival independent of trophic support. Caffeine has been shown to translocate Ca^{2+} from IP_3-insensitive, caffeine-sensitive stores in sympathetic neurons [32,33]. Caffeine, however, failed to elicit transmitter release in these cells [32]. It remains thus to be determined whether or not Ca^{2+} release from IP_3-sensitive or other sites may promote neuronal survival. In this regard, muscarinic cholinergic stimulation, which produces a remarkable release of IP_3 [30,33], fails to mobilize free Ca^{2+} [33], and does not support neuronal survival of superior cervical ganglion cells [6]. Thus, it is rather safe to conclude that a transient rise of $[Ca^{2+}]_i$, irrespective of internal or external origin of Ca^{2+}, is not sufficient for neuronal survival. Continuous supply of Ca^{2+}, possibly from Ca^{2+} flux through plasma membrane, would be required for neurons to keep sustained levels of $[Ca^{2+}]_i$. However, the specific mechanism of the saving effect of sustained levels of $[Ca^{2+}]_i$ remains unexplained.

The time-course of cAMP accumulation in response to caffeine is worth mentioning. The initial phase of the caffeine action involves rapid recovery of the cAMP level of NGF-deprived neurons to normal level in the presence of NGF; the second phase includes gradual accumulation of the cAMP level, reaching a plateau by 10 h followed by a small decrease by 24 h. Because elevated cAMP levels caused by treatment with caffeine varied in time, it is rather difficult to estimate the exact levels of cAMP required for neuronal survival. Thus, it remains to be determined as to whether normal level ($\sim$ 0.40 pmol/well) is sufficient or more elevation in the cAMP level is necessary. However, caffeine promoted the survival of NGF-deprived neurons only if neurons were continuously exposed for more than 10 h. It is tempting to speculate that elevation of cAMP to more than the normal level would be necessary. This study also provided evidence that a transient rise of cAMP did not support neuronal survival. Wakade et al. [33] also describes that sustained cAMP level is required for neuronal survival of chick sympathetic neurons.

The role of cAMP in mediating the response of sympathetic neurons or PC12 cells to NGF [34] is still controversial. There are some reports that treatment which increases cAMP level mimics some of the effect induced by NGF treatment [35,36]. Some reports show that NGF and cAMP do not act additively when they present in saturating amounts, suggesting that they act through a common pathway. The evidence presented here shows that acute withdrawal of NGF resulted in a decrease in cAMP levels, suggesting that NGF controls the level of cAMP in sympathetic neurons. It is worthy to note that the basal level of $[Ca^{2+}]_i$ remained the same in response to NGF deprivation in these neurons [6], suggesting that the levels of cAMP and $[Ca^{2+}]_i$ are regulated separately in these cells. Evidence is accumulating which suggests that the saving effect of increased cAMP level may be different from the trophic effect of NGF [10,11,25,37,38]. Additivity of some effects of NGF and cAMP has been interpreted as indicating that NGF acts through a cAMP-independent pathway [39]. Rydel and Greene [12] reported that cAMP analogs promote the survival of the same neuronal population as that maintained by NGF in cultured sympathetic neurons, but unlike NGF, the cAMP analogues do not evoke somatic hypertrophy. These findings suggest that NGF and cAMP promote neuronal survival via distinct mechanisms. Based on these findings, we speculate that cAMP-promoted survival may require the elevated level of cAMP ($\sim$ 0.6 pmol/well), which is approx. 150% higher than that of cells maintained in the presence of NGF.

There is good evidence to show that presynaptic electrical stimulation of superior cervical ganglia leads to the accumulating increase in cellular cAMP level, suggesting that presynaptic input may mediate cascade(s) of events involving cAMP [13]. Agents inducing elevated levels of cAMP may exert their trophic effects through activation of this cascade. It is also possible that elevated levels of cAMP suppress the active cell death program proposed by Martin et al.

[20]. The lag period of 5 h between the cycloheximide-sensitive step and the caffeine-sensitive step in this cascade shows that the drug blocks the expression of this cascade post-translationally, and suggests the presence of other step(s) involved, since simple diffusion of the product of cycloheximide-sensitive step does not explain such a big time difference. The modification of the product would follow during this time period. The mechanism of action of elevated levels of cAMP needs to be clarified. Edwards et al. [40] also observed that cAMP may rescue neuronal death by acting post-translationally.

In summary, these findings indicate that elevated levels of second messengers including not only $[Ca^{2+}]_i$, but also cAMP level, support neuronal survival of superior cervical ganglion cells in vitro in the absence of trophic factor support.

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References

1 Phillipson, O.T. and Sandler, M. (1975) Brain Res. 90, 278–281.
2 Furber, S., Oppenheim, R.W. and Prevette, D. (1987) J. Neurosci. 7, 1816–1832.
3 Lipton, S.A. (1986) Proc. Natl. Acad. Sci. USA 83, 9774–9778.
4 Maderdrut, J.L., Oppenheim, R.W. and Prevette, D. (1988) Brain Res. 444, 189–194.
5 Collins, F. and Lile, J.D. (1989) Brain Res. 502, 99–108.
6 Koike, T., Martin, D.P. and Johnson, E.M., Jr. (1989) Proc. Natl. Acad. Sci. USA 86, 6421–6425.
7 Gallo, V., Kinsbury, A., Balazs, R. and Jorgensen, R.O. (1987) J. Neurosci. 7, 2203–2213.
8 Koike, T. and Tanaka, S. (1991) Proc. Natl. Acad. Sci. USA 88, 3892–3896.
9 Collins, F., Schmidt, M.F., Guthrin, P.B. and Kater, S. B. (1991) J. Neurosci. 11, 2582–2587.
10 Levy, B.K., Martin, D.P. and Johnson, E.M., Jr. (1988) Soc. Neurosci. Abst. 14, 1118.
11 Martin, D.P., Wallace, T.L. and Johnson, E.M., Jr. (1990) J. Neurosci. 10, 184–193.
12 Rydel, R.E. and Greene, L.A. (1988) Proc. Natl. Acad. Sci. USA 85, 1257–1261.
13 Bigges, C.A., Whiting, G.J., Ariano, M.A. and McAfee, D.A. (1982) Cell. Mol. Neurobiol. 2, 129–141.
14 Brown, D.A., Caulfield, M.P. and Kirby, P.J. (1979) J. Physiol. 290, 441–451.
15 Lipscombe, D., Madison, D.V., Peonie, M., Reuter, H., Tsien, R.W. and Tsien, R.Y. (1988) Neuron 1, 355–365.
16 Thayer, T.A., Hirning, L.D. and Miller, R.J. (1989) Mol. Pharmacol. 34, 664–673.
17 Walicke, P.A. and Patterson, P.H. (1981) J. Neurosci. 1, 333–342.
18 Johnson, M.I. and Argiro, V. (1983) Methods Enzymol. 103, 334–347.
19 MaCarthy, K.D. and Partlow, L.M. (1976) Brain Res. 114, 391–414.
20 Martin, D.P., Schmidt, R.E., DiStefano, P.S., Lowry, O. H., Carter, J.G. and Johnson, E.M., Jr. (1988) J. Cell Biol. 106, 829–844.
21 Grynkiewicz, G., Peonie, M. and Tsien, R.Y. (1985) J. Biol. Chem. 260, 3440–3450.
22 Thayer, S.A., Hiring, L.D. and Miller, R.J. (1987) Mol. Pharmacol. 32, 579–586.
23 Kessler, J.A., Spray, D.C., Saez, J.C. and Bennett, M. V. (1984) Proc. Natl. Acad. Sci. USA 81, 6235–6239.
24 Mobley, W.C., Schenker, A. and Shooter, E.M. (1976) Biochem. 15, 5543–5551.
25 Rich, K.M., Yip, H.K., Osborne, P.A., Schmidt, R.E., and Johnson, E.M., Jr. (1984) J. Comp. Neurol. 230, 110–118.
26 Manthorpe, M., Skaper, S. and Varon, S. (1980) Brain Res. 196, 467–482.
27 Scotini, E., Carpenedo, F. and Fassina, G. (1983) Pharmacol. Res. Commun. 15, 131–143.
28 Stefanovich, V. (1979) Neurochem. Res. 4, 681–687.
29 Schwarzschild, M.A. and Zigmond, R.E. (1991) J. Neurochem. 56, 400–406
30 Audigier, S., Barberis, C. and Jard, S. (1986) Brain Res. 376, 363–367.
31 Lazarus, K.J., Bradshaw, R.A., West, N.R. and Bunge, R.P. (1976) Brain Res. 113, 334–347.
32 Wakade, T.D., Bhave, S.V., Bhave, A., Przywara, D.A., and Wakade, A.R. (1990) J. Neurochem. 55, 1806–1809.
33 Wakade, T.D., Bhave, A.S., Bhave, S.V. and Wakade, A. R. (1991) Blood Vessels 28, 6–10.
34 Green, L.A. and Shooter, E.M. (1980) Ann. Rev. Neurosci. 3, 353–402.
35 Gunning, P.W., Landreth, G.E., Bothwell, M.A. and Shooter, E.M. (1981) J. Cell Biol. 89, 240–245.
36 Richter-Landsberg, C. and Jastorff, B. (1986) J. Cell Biol. 102, 821–829.
37 Suidan, H.S., Murrell, R.D. and Tolkovsky, A.M. (1991) Cell Regulation 2, 13–25.
38 Wakade, A.R., Bhave, S.V., Malhotra, R.K. and Wakade, T.D. (1990) J. Neurochem. 54, 1281–1287.
39 Skaper, S.D. and Varon, S. (1984) J. Neurochem. 42, 116–122.
40 Edwards, S.N., Buckmaster, A.E. and Tolkovsky, A.M. (1991) J. Neurochem. 57, 2140–2143.