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Nimodipine and flunarizine have different effects on survival and morphology of PC12 cells during nerve growth factor deprivation

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Abstract

The purpose of this study was to examine the effect of antagonists of different subtypes of Ca^{2+} channels (nimodipine and flunarizine) and two types of Ca^{2+} chelating agents (the cell permeant Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid acetoxymethylester (BAPTA-AM) and the cell non-permeant Ca^{2+} chelator EGTA) on neurite retraction and cell death of nerve growth factor (NGF)-differentiated PC12 cells after NGF deprivation. We demonstrated that flunarizine and nimodipine, but not BAPTA-AM and EGTA, provided protection against cell death due to NGF deprivation. Using time-lapse videomicroscopy and quantitative image analysis, we found that retraction of neurites was an early and fast phenomenon after removal of NGF. None of the compounds tested (flunarizine, nimodipine, BAPTA-AM, EGTA) could prevent the retraction of neurites.

Keywords: Ca^{2+}; PC12 cell; NGF; Cell death; Neurite

1. Introduction

During embryonic development, many more neurons are made than are needed, and approximately half of these neurons are subsequently deleted. This seemingly wasteful process of neuronal death during development is thought to be a mechanism whereby the size of each neuronal population is matched to the size of the target tissue to be innervated. Developing neurons compete for, and are dependent on, target-derived neurotrophic factors such as nerve growth factor (NGF). Insufficient access to trophic factor initiates a protein and RNA synthesis-dependent degenerative process that terminates in neuronal death (Martin et al., 1988; Oppenheim et al., 1990; Scott and Davies, 1990). NGF is the best characterized of the neurotrophic substances. It promotes the survival, growth and differentiation of neuronal populations in both the peripheral and the central nervous system (Levi-Montalcini, 1987), but the mechanism of these actions is only partially understood (Levi and Alema, 1991; Batistatou et al., 1992). PC12 cells, a rat pheochromocytoma cell line (Greene, 1978), have been successfully used for studying the survival-promoting actions of NGF and the mechanism of apoptotic neuronal death after withdrawal of trophic support (Batistatou and Greene, 1991; Rukenstein et al., 1991). In serum-containing medium, PC12 cells divide and display many characteristics of adrenal chromaffin cells. Within several days of NGF exposure, these cells stop dividing and acquire numerous properties of mature sympathetic neurons including extension of neurites (Greene, 1978), electrical excitability, and expression of neuronal proteins (Burstein and Greene, 1978). When cultured in serum-free medium without NGF, both naive and neuronally differentiated PC12 cells die via an apoptotic mechanism (Greene and Tischler, 1976; Batistatou and Greene, 1991; Rukenstein et al., 1991; Mesner et al., 1992).

A variety of studies implicate Ca^{2+} in the regulation of apoptosis (McConkey and Orrenius, 1995). The influx of extracellular Ca^{2+} appears to play a critical role in neuronal degeneration due to the toxicity of excitatory amino acids (Choi, 1988) or after glucose deprivation (Cheng and Mattson, 1991, 1992). Although Ca^{2+} appears to be a

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factor in cell death, its role in neuronal death during development or after trophic factor deprivation is not known (Eichler et al., 1992).

In this study, we tested agents that could protect PC12 cells against NGF deprivation. The agents tested included drugs interfering with different subtypes of Ca^{2+} channels, namely nimodipine and flunarizine belonging to the dihydropyridine and diphenylpiperazine families (Vanhoutte and Paoletti, 1987), and Ca^{2+} chelating agents such as the cell permeant 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid acetoxymethylester (BAPTA-AM) and the cell non-permeant EGTA. The effect of these compounds on $[Ca^{2+}]_i$, neurite length and cell viability was studied.

2. Materials and methods

2.1. Cell culture

PC12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, GIBCO BRL) supplemented with 7.5% horse serum (GIBCO BRL), 7.5% fetal calf serum (HyClone), 2 mM L-glutamine (GIBCO BRL) and 0.5 μg/ml gentamycin (GIBCO BRL), in a humified incubator aerated with 5% CO_2 in air at 37°C.

2.2. Viability measurements

For the viability measurements in 96-well plates, 2×10^4 PC12 cells were seeded per poly-L-lysine-coated well in 200 μl DMEM supplemented with 7.5% horse serum, 7.5% fetal calf serum and 2 mM L-glutamine. Four hours after plating the medium was replaced with DMEM supplemented with 1% horse serum, 2 mM L-glutamine and 50 ng/ml NGF (Sigma or Alomone labs). After 4 days of differentiation, flunarizine, nimodipine and BAPTA-AM (from 10^{-2} M stock solutions in dimethylsulfoxide (DMSO)) were tested at concentrations ranging between 10^{-5} and 10^{-7} M for their ability to prevent neuronal death after NGF deprivation. After a 4-h pretreatment, the medium was changed to DMEM containing 50 ng/ml NGF or to DMEM supplemented with the various concentrations of flunarizine, nimodipine or BAPTA-AM. Control cells received the same amount of DMSO.

For the viability experiments using EGTA, the medium was changed after 4 days of differentiation to DMEM or DMEM supplemented with 50 ng/ml NGF containing 1, 5 or 10 mM EGTA. For the control situation DMEM or DMEM with 50 ng/ml NGF was used. EGTA was dissolved in DMEM, the pH was adjusted to 7.4, and the solution was made sterile using a Sterivex-GS filter unit (Millipore). Cell viability was determined 24 h later by adding 100 μl of a 1 mg/ml XTT (2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2 *H*-tetrazolium-5-carboxanilide, Sigma) solution in DMEM (37°C) supplemented with 0.02 mM phenazine methosulfate (Sigma). The plates were then incubated at 37°C for 2.5 h. After this time the optical densities were read (Molecular devices) at 450 nm, using the value at 650 nm as a reference. The XTT assay is based on the conversion of XTT into formazan, the red-colored reduced form of XTT.

2.3. $[Ca^{2+}]_i$ assessed with Fura-2 ratio measurements

To assess $[Ca^{2+}]_i$, PC12 cells were loaded with the Ca^{2+} indicator dye Fura-2 AM (Molecular Probes, Leiden, the Netherlands) as described previously (Dispersyn et al., 1998). Briefly, cells were loaded with 10 μM Fura-2 AM (from a 1-mM stock solution in DMSO) for 30 min at 37°C in phosphate-buffered saline (PBS, GIBCO BRL) containing 0.005% F127 (Molecular Probes). $[Ca^{2+}]_i$ was evaluated at room temperature, on the stage of a Zeiss Axiovert 35 inverted microscope, by measuring the ratio (r) of Fura-2 emission (520 nm) when excited alternately at 340 nm and 380 nm through high-quality bandpass filters on a sliding actuator. Emitted fluorescence was collected with an intensified CCD camera (Photonic Science). The video output was connected to the VINO analogue video input port of an Indy Workstation (Silicon Graphics, USA). The fluorescence intensity of each cell in the image was calculated after segmentation by SCIL-Image software (TNO, Delft, The Netherlands) and stored on disk.

2.4. Time-lapse video microscopy

Images were captured on a Zeiss Axiovert 35 using a MXR camera. A computer-driven motorized stage (Merzhauser, Germany) was used to record several sets of (x, y, z) coordinates corresponding to the fields of interest. Movies were composed by driving the stage to the fields, executing an autofocus routine, grabbing the image and appending it to the movie-file of the current field (SCIL-image analysis software, TNO).

2.5. Quantification of neurite length

PC12 cells were plated in poly-L-lysine (Sigma)-coated 12-well plates. To each well 5×10^4 cells in 1.5 ml DMEM supplemented with 7.5% horse serum, 7.5% foetal calf serum, 2 mM L-glutamine and 0.5 μg/ml gentamycin were added. To differentiate the cells, medium was changed 4 h later to DMEM supplemented with 1% horse serum, 2 mM L-glutamine and 50 ng/ml NGF. After 4 days of differentiation the experiments were started by pretreating the NGF-differentiated cells for 4 h with different concentrations of flunarizine or nimodipine (10^{-6}–10^{-8} M) and BAPTA (10^{-5}–10^{-7} M). After this pretreatment the medium was changed to DMEM supplemented with 50 ng/ml NGF or to DMEM, each supplemented with differ-

ent concentrations of the compounds. After 24 h the cells were fixed with 1% glutaraldehyde for 10 min. Then the cells were washed twice with distilled water and the plates were dried in an incubator. The plates were observed on an Axiovert 10 (Zeiss, Germany) using bright field illumination. The images were captured by a MX5-CCD (HCS, The Netherlands) camera and digitized by an Indy workstation (Silicon Graphics). The analysis was performed as follows. Sixty-four neatly aligned images, forming a 8×8 matrix of images, were selected and contained about 3000 cells. The analysis software automatically detected cell bodies and neurites. Cell body size and the length of each of the individual neurites were saved on file. For time-lapse phase contrast videomicroscopy, manual analysis was performed by projecting the digitally stored images on the computer graphic screen. The neurites were interactively traced (using a mouse-driven cursor as a pointer) and the length estimation was performed by perimeter calculation of the traced curve (SCIL Image 1.3, TNO). For the quantification of neurite lengths in low extracellular Ca^{2+} medium with EGTA, the same experimental setup was used as described above except that, in this case, cells were not pretreated with EGTA.

3. Results

3.1. Effect of nimodipine, flunarizine, BAPTA-AM and EGTA on $[Ca^{2+}]_i$

The effect of nimodipine, flunarizine, BAPTA-AM and EGTA on intracellular Ca^{2+} levels in NGF-differentiated PC12 cells was assessed using the fluorescent Ca^{2+}-sensitive dye Fura-2. Fura-2 ratios (340/380 nm) were measured in individual cells in PBS for 5 min. Then the compound to be tested was added and Fura-2 ratios were measured for another 50 min. The results were plotted as ratios for the individual cells, calculated by dividing the Fura-2 ratio (340/380 nm) measured after a 50-min treatment with the compound by the basal Fura-2 ratio of the same cell obtained at the start of the experiment. In Fig. 1 the ratios from one representative experiment are shown ($n =$ 31–75 cells). Nimodipine (Fig. 1A) and flunarizine (Fig. 1B) did not significantly decrease $[Ca^{2+}]_i$ of NGF-differentiated PC12 cells after the 50-min treatment, whereas BAPTA-AM at 10^{-5} M (Fig. 1C) and EGTA at 1, 5 and 10 mM (Fig. 1D) significantly decreased the normalized ratios, which is indicative of a reduction in $[Ca^{2+}]_i$ (Dunnett's method, $P < 0.01$).

3.2. Effect of BAPTA-AM, flunarizine, nimodipine and EGTA on survival of NGF-deprived PC12 cells

Using the XTT assay, we determined the ability of the compounds to prevent cell death induced by NGF withdrawal from PC12 cells that had been differentiated by pre-exposure to NGF in low-serum medium for 4 days. Differentiated PC12 cells die after NGF withdrawal, with 20% to 40% of the cells being dead after 24 h. In Fig. 2, the survival of cells treated with flunarizine and nimodipine is plotted relative to the control where cells were kept in DMEM containing 50 ng/ml NGF. Using the Jonckheere–Terpstra test for statistical analysis of the dose–response relationship of the compounds, the following two-sided P-values were found: 0.008 for flunarizine, 0.0163 for nimodipine and 0.9164 for BAPTA-AM. Hence flunarizine and nimodipine, but not BAPTA-AM, provided pro-

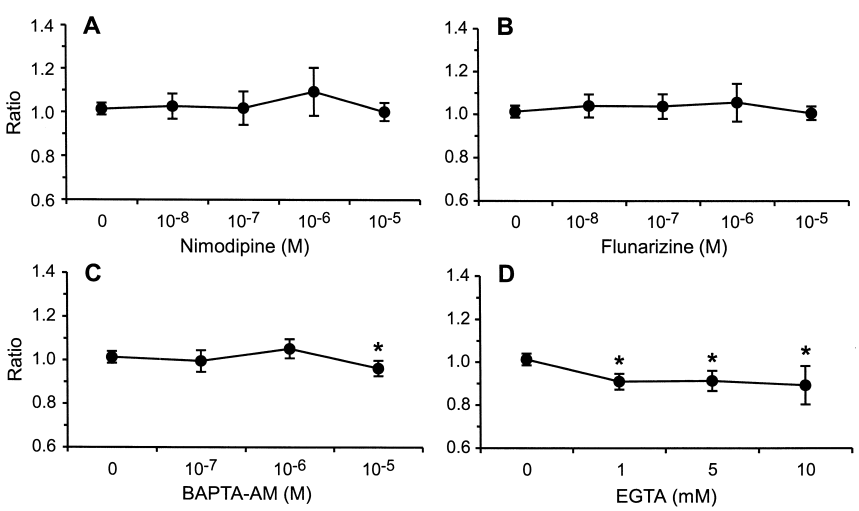

Fig. 1. Effect of nimodipine (A), flunarizine (B), BAPTA-AM (C) and EGTA (D) on $[Ca^{2+}]_I$, as assessed with Fura-2. Fura-2 ratios (340/380 nm) of individual cells obtained after a 50-min treatment were normalized to the Fura-2 ratios obtained before application of the compound. Values represent the means ± S.D. ($n = 31–75$ cells) from one representative experiment. $^*P < 0.01$, significantly lower than solvent control (Dunnett's method).

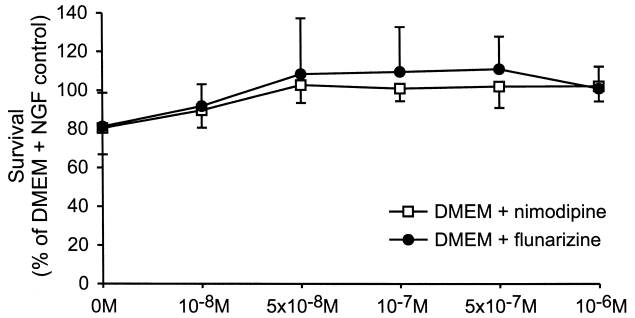

Fig. 2. Effect of nimodipine and flunarizine on cell viability of NGF-differentiated PC12 cells after 24 h of NGF-deprivation, as assessed by XTT assay. Values represent the means ± S.D. ($n = 12$ or 15) from four independent experiments and are plotted relative to the control value (cells in DMEM containing 50 ng/ml NGF). The Jonckheere–Terpstra test was used for statistical analysis of the dose–response relationship of the compounds. The two-sided *P*-values are 0.008 for flunarizine and 0.0163 for nimodipine.

tection against cell death induced by NGF deprivation as measured by XTT.

To assess the role of extracellular Ca^{2+} in cell death after NGF deprivation, PC12 cells grown in the presence of NGF for 4 days were incubated in medium containing a very low Ca^{2+} concentration. Three concentrations of EGTA (1, 5 or 10 mM) were added to DMEM or DMEM with 50 ng/ml NGF to obtain low Ca^{2+} conditions. As is shown in Table 1, depriving PC12 cells of NGF resulted in a ~40% cell loss after 24 h. NGF-differentiated PC12 cells, when deprived in the presence of 1 mM EGTA, showed a similar death rate. Five and ten millimolar EGTA resulted in an additional 10% cell death.

3.3. Effect of NGF deprivation on $[Ca^{2+}]_i$ of PC12 cells

Intracellular Ca^{2+} levels in PC12 cells were assessed with the use of the fluorescent Ca^{2+}-sensitive dye Fura-2. NGF-differentiated PC12 cells were deprived of NGF by changing the medium to DMEM, or DMEM supplemented with 50 ng/ml NGF for control cells. Twenty-four hours later Fura-2 ratios (340/380 nm) were measured in individual cells. Fura-2 ratios were 0.51 ± 0.05 (mean ± S.D., $n = 25$ cells in two independent experiments) in PC12 cells maintained with NGF and 0.55 ± 0.09 (mean ± S.D., $n = 15$ cells in two independent experiments) in PC12 cells deprived of NGF. From these data, we may conclude that NGF deprivation has no effect on the basal Ca^{2+} levels in PC12 cells.

3.4. Effect of NGF deprivation on morphology of PC12 cells

Time-lapse phase-contrast videomicroscopy was performed to assess the effects of NGF deprivation on the morphology of NGF-differentiated PC12 cells. NGF-treated and NGF-deprived PC12 cells were observed with time after the medium was changed from DMEM supplemented with 1% horse serum and 50 ng/ml NGF to DMEM supplemented with NGF or to DMEM.

The phase-contrast micrographs (Fig. 3) show the morphology of the cells with time after the medium was changed. The neurite length of the cells was determined manually from the phase contrast time lapse recordings. Three neurites of NGF-treated and three neurites of NGF-deprived cells (three cells in each condition) were measured. In Fig. 4A the lengths of these neurites are presented relative to the neurite length at the time when the medium was changed. This graph shows that when a neurite started to retract upon NGF deprivation, neurite length decreased rapidly (within 5–30 min). The neurites of NGF-deprived PC12 cells retracted, whereas the neurites of NGF-treated PC12 cells remained intact, although there were some fluctuations in the lengths of the latter.

To further examine the loss of neuronal architecture with time after NGF deprivation, we used quantitative light microscopy: PC12 cells plated in 12-well plates at 50,000 cells/well differentiated in the presence of NGF for 4 days and then the medium was changed to DMEM or DMEM with NGF. At different time points between 0 and 48 h after the medium was changed, the cells were fixed with 1% glutaraldehyde. After software analysis of the captured bright field images, neurite lengths were obtained. In Fig. 4B neurite lengths are presented relative to the neurite length at the time when the medium was changed. NGF deprivation already caused a reduction in total neurite length of ~16% after 1 h. In the next hour the neurite length of NGF-deprived PC12 cells decreased another 10%. At around 3–4 h of deprivation the neurite length had reached a value of ~70% of the original neurite length. After 22 h of NGF deprivation the total neurite length had decreased to 40% of the control (DMEM containing 50 ng/ml NGF). No significant change was observed in the neurite length of NGF-treated PC12 cells.

Table 1
Effect of EGTA on cell viability of NGF-deprived PC12 cells ($t = 24$ h). Cell viability of NGF-differentiated PC12 cells was determined by the XTT assay, 24 h after the medium was changed for DMEM or DMEM + 50 ng/ml NGF, supplemented with different concentrations of EGTA. Values represent the means ± S.D. ($n = 60$) of two independent experiments and are expressed as percentages of survival in the control situation (cells in DMEM containing 50 ng/ml NGF)

Concentration of EGTA in mM	DMEM + NGF (% of DMEM + NGF control)	DMEM (% of DMEM + NGF control)
0	100 ± 0	63.8 ± 10.1
1	107.0 ± 16.6	61.1 ± 10.4
5	106.1 ± 18.3	52.5 ± 4.1[a]
10	100.6 ± 19.0	53.0 ± 6.5[a]

[a] Statistically different from control (Dunnett's method, $P < 0.05$).

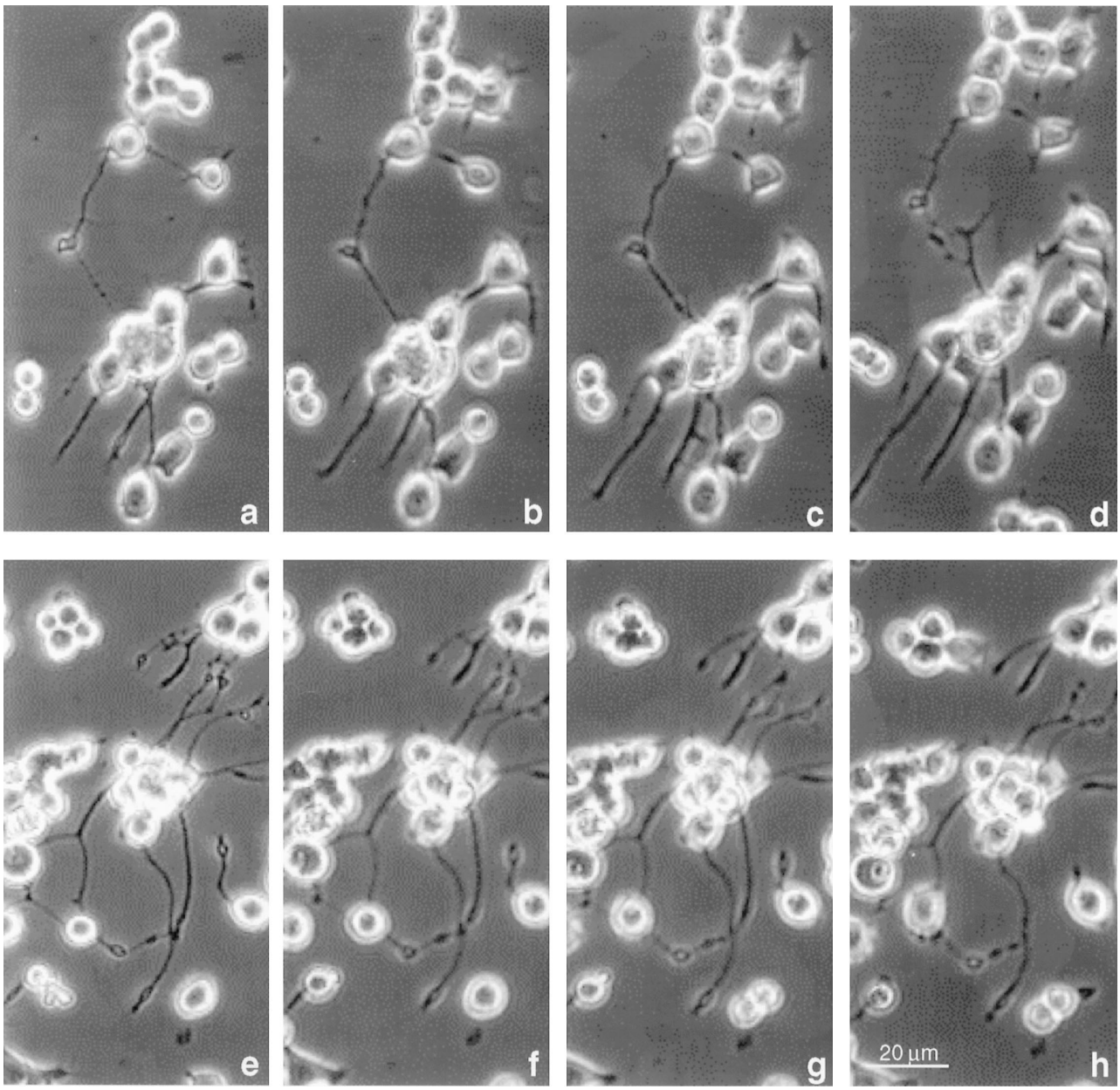

Fig. 3. Effect of NGF-deprivation on the morphology of NGF differentiated PC12 cells as assessed by time lapse phase contrast videomicroscopy. The phase contrast micrographs show the morphology the NGF-differentiated PC12 cells at 0 (a,e), 0.5 (b,f), 1 (c,d) and 2 h (d,h) after the medium was changed for DMEM supplemented with 50 ng/ml NGF (a–d) and DMEM (e–h). Arrowheads indicate neurites that retracted during NGF deprivation.

3.5. Effect of BAPTA-AM, flunarizine and nimodipine on neurite retraction during NGF deprivation

We wanted to know whether the compounds flunarizine and nimodipine, which protect differentiated PC12 cells from death induced by NGF deprivation, also protected the cells from NGF withdrawal-induced neurite retraction. NGF-differentiated PC12 cells were first pretreated with the various concentrations of BAPTA-AM, flunarizine and nimodipine. As is shown in Fig. 5, neurite lengths were plotted relative to the neurite length obtained for the control NGF-treated PC12 cells after 24 h. Nimodipine (Fig. 5A) and flunarizine (Fig. 5B) applied at 10^{-6}, 10^{-7} or 10^{-8} M did not significantly inhibit neurite retraction in NGF-differentiated PC12 cells during 24 h of NGF deprivation.

The total neurite length of cultures treated with BAPTA-AM (Fig. 5C) (10^{-5}, 10^{-6} or 10^{-7} M) was not different from that in the NGF deprivation control, whereas the total neurite length of NGF control cells (DMEM + NGF) was decreased by $\sim$30% at 10^{-5} M BAPTA. Using the Jonckheere–Terpstra test for statistical analysis

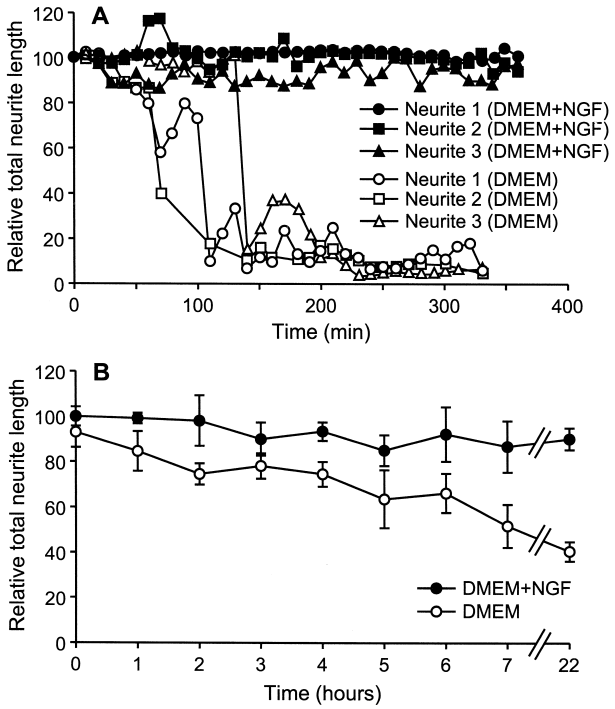

Fig. 4. Effect of NGF deprivation on total neurite length of the NGF-differentiated PC12 cells. (A) Neurite lengths were scored manually from the phase-contrast time-lapse recordings. Three neurites of NGF-treated and three neurites of NGF-deprived PC12 cells were monitored from the time after the medium was changed for DMEM supplemented with NGF or DMEM, respectively. Neurite lengths of NGF control cells and NGF-deprived cells are presented as values relative to the neurite length at the time when the medium was changed. (B) The effect of NGF deprivation on neurite length was quantified. At different time points after the medium was changed, the cells in 12-well plates were fixed with 1% glutaraldehyde. After software analysis of the captured bright field images, neurite lengths were determined. Values represent the means ± S.D. ($n = 10$) and are expressed as percentages of the neurite length in the control situation (cells in DMEM containing 50 ng/ml NGF at the time the medium was changed).

of the dose–response relationship of the compounds during NGF deprivation, the following non-significant two-sided *P*-values were found: 0.18 for flunarizine, 0.24 for nimodipine and 0.03 for BAPTA-AM toxicity in DMEM + NGF condition. The results obtained from these experiments demonstrate that neither flunarizine nor nimodipine affected the retraction of neurites. BAPTA-AM, an intracellular Ca^{2+} chelator, also could not prevent neurite retraction during NGF deprivation. In the presence of NGF, BAPTA-AM (10^{-5} M) itself caused neurite retraction.

3.6. Effect of EGTA on neurite retraction during NGF deprivation

In order to evaluate the contribution of extracellular Ca^{2+} to changes in morphology during NGF deprivation, neurite length was measured in the presence of the extracellular Ca^{2+} chelator EGTA (0, 1, 5 or 10 mM). In Table 2 neurite lengths are presented relative to the length of the neurites in control NGF-treated PC12 cells (at $t = 24$ h). Depriving differentiated PC12 cells of NGF for 24 h reduced the relative neurite length by ~60%. First, we examined whether EGTA had an effect on the morphology of NGF-treated PC12 cells. At 1 mM EGTA no change in neurite length occurred when compared to the control situation without EGTA. After 24 h the neurite length of NGF-treated cells in medium supplemented with 1 mM EGTA was 106% of that of the control NGF-treated cells. At 5 or 10 mM EGTA, neurite length significantly de-

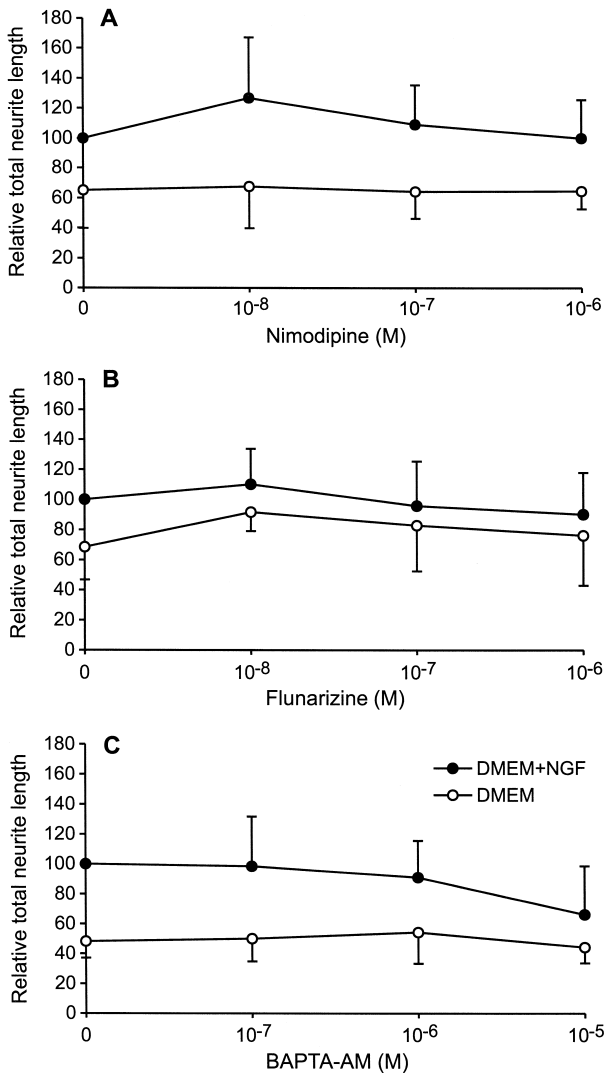

Fig. 5. Effect of nimodipine (A), flunarizine (B) and BAPTA-AM (C) on the morphology of NGF-differentiated PC12 cells after 24 h of NGF deprivation. Values represent the means ($6 \geq n \leq 10$) from three independent experiments and are expressed as percentages of the neurite length of the cells in the control situation (cells in DMEM containing 50 ng/ml NGF). Using the Jonckheere–Terpstra test for the statistical analysis of the dose–response relationship of the compounds during NGF deprivation, the following two-sided *P*-values were found: 0.18 for flunarizine, 0.24 for nimodipine and 0.03 for BAPTA-AM toxicity in DMEM + NGF condition.

Table 2
Effect of EGTA on neurite length of NGF-deprived PC12 cells ($t = 24$ h). Total neurite length of NGF-differentiated PC12 cells was determined using automatic quantitative light microscopy, 24 h after medium was changed for DMEM or DMEM + 50 ng/ml NGF, supplemented with different concentrations of EGTA. Values represent the means ± S.D. ($n = 10$) and are expressed as percentages of the neurite length of cells in the control situation (cells in DMEM containing 50 ng/ml NGF)

Concentration of EGTA in mM	DMEM + NGF (% of DMEM + NGF control)	DMEM (% of DMEM + NGF control)
0	100 ± 0	37.5 ± 7.3
1	106.9 ± 27.4	33.5 ± 6.2
5	13.5 ± 7.0[a]	13.0 ± 6.0[a]
10	11.1 ± 4.5[a]	17.2 ± 9.4[a]

[a] Statistically different from control (Dunnett's method, $P < 0.05$).

creased after 24 h to 13% and 11% of control (Dunnett's method, $P < 0.05$). The neurite length of PC12 cells that had been deprived of NGF for 24 h in the presence of 1 mM EGTA decreased by 67%. This means that 1 mM EGTA was not capable of protecting the cells against neurite retraction after NGF deprivation. When the medium was changed to DMEM supplemented with 5 and 10 mM EGTA, the neurite length was reduced by ~88% after 24 h relative to that of the NGF-treated cells. This means that addition of 5 or 10 mM EGTA significantly reduced neurite length during NGF deprivation compared to the effect of NGF deprivation without EGTA (Dunnett's method, $P < 0.05$).

4. Discussion

The purpose of this study was to examine the effect of Ca^{2+} channel antagonists and Ca^{2+} chelating agents on the morphology and survival of NGF-deprived PC12 cells. We showed that neurite retraction in PC12 cells after NGF deprivation is an early phenomenon, whereas cell death occurs late, i.e., 24–48 h after the onset of deprivation. After 24 h of NGF deprivation there was a 60% decrease in neurite length vs. 20%–30% cell death. Further, we demonstrated that PC12 cells can be protected against cell death induced by NGF deprivation by flunarizine and nimodipine, whereas none of these compounds could prevent the retraction of neurites during NGF deprivation. EGTA or BAPTA-AM neither protected against cell death nor prevented retraction of neurites as a result of NGF deprivation. Although treatment with EGTA or BAPTA-AM had a different effect on the survival and morphology of NGF-differentiated PC12 cells in the control situation (DMEM + NGF), at high concentrations EGTA and BAPTA-AM had little effect on cell survival, whereas the total neurite length measured in these conditions was decreased. Furthermore, we showed that after a 50-min treatment only BAPTA-AM (10^{-5} M) and EGTA (1, 5 and 10 mM) induced a reduction in $[Ca^{2+}]_i$.

Intracellular Ca^{2+} homeostasis is regulated by Ca^{2+}-binding proteins, the endoplasmic reticulum, mitochondria, as well as by at least two plasmalemmal extrusion molecules, the Na^+/Ca^{2+} exchanger and Ca^{2+}-ATPase (Miller, 1991). Disturbances of one of these interactions can result in an enhanced Ca^{2+} influx, release of Ca^{2+} from intracellular stores, and/or inhibition of Ca^{2+} extrusion through the plasma membrane. This may lead to an uncontrolled, sustained rise in $[Ca^{2+}]_i$, which can potentially activate Ca^{2+}-dependent degradative enzymes, such as proteases, phospholipases, endonucleases, or cause mitochondrial damage (Nicotera et al., 1992). Sustained increases in $[Ca^{2+}]_i$ caused by a variety of insults are postulated to be a major mechanism of neuronal death by either necrosis or apoptosis (Meldrum and Garthwaite, 1990; Nicotera et al., 1992; Lipton and Rosenberg, 1994; Dowd, 1995; Trump and Berezesky, 1995). EGTA is a Ca^{2+} chelating agent and its presence in the extracellular medium should deplete Ca^{2+} from the medium and reduce cytosolic Ca^{2+} levels (Lindenboim et al., 1995). In NGF-differentiated PC12 cells deprived of NGF, addition of 1 mM EGTA had no effect on cell survival vs. control, whereas 5 and 10 mM EGTA increased cell death by 10%. We showed that lowering the extracellular Ca^{2+} concentration failed to protect against cell death caused by growth factor deprivation. This is consistent with an earlier study (Eichler et al., 1994) showing that neurons deprived of NGF in the virtual absence of extracellular Ca^{2+} still underwent neuronal death in a manner similar to NGF-deprived neurons grown in medium containing a standard concentration of extracellular Ca^{2+}. Lowering the extracellular Ca^{2+} concentration by adding EGTA to the medium could not prevent the retraction of neurites caused by NGF deprivation. At higher concentrations, it even caused an additional reduction in neurite length.

The cytosolic Ca^{2+} buffering capacity, i.e., the ability to bind a large fraction of the Ca^{2+} pool contained in the cytosolic compartment, plays an important role in Ca^{2+} homeostasis (Clementi et al., 1996). BAPTA-AM is a lipophilic compound capable of crossing cell membranes. It has been reported that cell-permeant Ca^{2+} chelating agents such as BAPTA-AM (at concentrations of 30–100 μM) can protect neurons from neurodegeneration induced by excessive excitatory stimulation in vitro (Tymianski et al., 1993, 1994) and by cerebral ischemia in vivo (Tymianski et al., 1994). In our study BAPTA-AM was not protective against neurite retration, or against cell death caused by NGF deprivation. We found that BAPTA-AM (at a high concentration) induced the retraction of neurites of NGF-differentiated PC12 cells in the presence of NGF (DMEM + NGF control), whereas it had no effect on survival (data not shown). In a recent study, it was reported that thapsigargin-induced apoptotic cell death in GT1-7 cells could not be prevented either by EGTA or by BAPTA-AM (Wei et al., 1998). Although Ca^{2+} influx from the extracellular space is often correlated with apop-

totic cell death, our results, in line with other observations (Martin et al., 1994; Dowd, 1995; Kroemer et al., 1995), suggest that this is not an essential requirement.

The above data demonstrate that both nimodipine and flunarizine prevent the death of NGF-deprived PC12 cells, but fail to protect against neurite retraction. The mechanism by which flunarizine provides protection both in vivo and in vitro is not known (Eichler et al., 1994). Flunarizine is classified as a class IV Ca^{2+} channel antagonist, non-selective for slow Ca^{2+} channels (Vanhoutte and Paoletti, 1987). Thomas and Seelig (1993) have demonstrated that flunarizine and other class IV Ca^{2+} channel antagonists have no proven specific binding to the voltage-gated (L-type) slow Ca^{2+} channel. We found that flunarizine did not change $[Ca^{2+}]_i$ in NGF-differentiated PC12 cells after a 50-min treatment. However, flunarizine is known to inhibit the transient increase in $[Ca^{2+}]_i$ after K^+-induced depolarization of the plasma membrane (Zhang et al., 1993; Villarroya et al., 1995). Rich and Hollowell (1990) found that flunarizine also affords neuronal protection by acting at an intracellular site, distinct from its blockade of voltage-dependent Ca^{2+} channels. It is suggested that such a site could be the mitochondria, where the lipophilic flunarizine might concentrate, thereby preventing the accumulation of Ca^{2+} and impairment of ATP synthesis (Eichler et al., 1994; Uceda et al., 1995). Recent evidence suggests that mitochondrial dysfunction plays a critical role in apoptosis (Mignotte and Vayssiere, 1998). Eichler et al. (1994) also reported that flunarizine may interact with Ca^{2+} and neuronal membranes to prevent lipid reorganization and subsequent membrane breakdown. Rich and Hollowell (1990) showed that flunarizine, but not nimodipine, prevents neuronal death in cultures of NGF-dependent embryonic sensory and sympathetic neurons after abrupt withdrawal of neurotrophic support. In our study not only flunarizine, but also nimodipine, a 1,4-dihydropyridine belonging to class III Ca^{2+} channel antagonists, was capable of providing protection against cell death after NGF deprivation. Nimodipine predominantly blocks L-type Ca^{2+} channels, which are present throughout the central nervous system. In this study no effect of nimodipine on $[Ca^{2+}]_i$ was observed in NGF-differentiated PC12 cells treated with 10^{-5}–10^{-8} M nimodipine for 50 min.

From our experiments it seemed that, 24 h after NGF deprivation, the basal Ca^{2+} levels in NGF-deprived PC12 cells (DMEM) were the same as those in NGF-treated cells (DMEM + NGF). However, this should be interpreted with caution, because the mean basal level for the NGF-deprived PC12 cells is the result for a selected subpopulation of cells that survived 24 h of NGF deprivation. The studies of Eichler et al. (1994) demonstrated that dorsal root ganglion neurons grown in the presence of NGF show a decline in $[Ca^{2+}]_i$ between 24 and 48 h after NGF deprivation. They found that agents that prevented neuronal death (flunarizine, cinnarizine) also prevented this decline in $[Ca^{2+}]_i$. Depolarization by high levels of extracellular K^+ promotes the survival of several types of neurons in vitro (Scott and Fisher, 1970; Koike et al., 1989; Collins et al., 1991; Franklin and Johnson 1992). The beneficial effect in vitro of high extracellular K^+ concentrations on NGF-deprived sympathetic neurons may be linked to influx of Ca^{2+} via dihydropyridine-sensitive L-type Ca^{2+} channels, resulting in elevated $[Ca^{2+}]_i$ (Koike et al., 1989). However, an increased $[Ca^{2+}]_i$ can induce apoptosis under some conditions (McConkey and Orrenius, 1995), and in the absence of NGF a high concentration of K^+ promotes the survival of sympathetic neurons without an increase in $[Ca^{2+}]_i$ (Murrell and Tolkovsky, 1993). According to Yu et al. (1997), high extracellular K^+ levels might attenuate neuronal apoptosis by reducing K^+ efflux. According to Teng and Greene (1993), elevated K^+ prevents the degeneration of neurites that occurs when NGF is withdrawn from PC12 cell cultures, probably by Ca^{2+} entry through voltage-sensitive L-type Ca^{2+} channels. They suggest that a decrease in $[Ca^{2+}]_i$ could be responsible for neurite degeneration after NGF withdrawal. We found that decreasing $[Ca^{2+}]_i$ by EGTA or BAPTA-AM caused retraction of the neurites of NGF-treated PC12 cells. EGTA even potentiated the retraction of neurites of NGF-deprived cells, whereas no additional effect of BAPTA-AM was found. Using the ratiometric Ca^{2+}-indicator Fura-2 AM, no changes in basal Ca^{2+} levels were detected during the first hours of NGF deprivation (data not shown). Taken together with our findings that neither BAPTA-AM, flunarizine, nimodipine nor EGTA could prevent neurite retraction, it is likely that the retraction of neurites of differentiated PC12 cells after withdrawal of NGF from the medium, which is an early and fast phenomenon, is not the result of an increase in $[Ca^{2+}]_i$. This is in line with the results of Reber and Bouron (1995). They showed that calyculin A (inhibitor of protein phosphatase 1 and 2) caused a fast but reversible change in cell morphology of differentiated PC12 cells, consisting of an initial retraction of neurites followed by a shape change of the cell bodies. They reported that the morphological changes occurred without obvious changes in $[Ca^{2+}]_i$. A rapid destruction of neuronal processes of human NT2N cells treated with calyculin A and okadaic acid (inhibitor of phosphatase 2A) was also reported by Merrick et al. (1997) and Nuydens et al. (1998).

A possible explanation for NGF deprivation-induced neurite retraction in the absence of Ca^{2+} changes is hyperphosphorylation of microtubule-associated-proteins (MAPs), in particular tau. The major function of the MAPs is stabilization of the microtubule system. Calyculin A was found to increase tau phosphorylation in NT2N by inhibiting the phosphatases, thereby decreasing the binding of tau to microtubules (Merrick et al., 1997). Nuydens et al. (1997) showed that in NGF-differentiated PC12 cells the level of aberrantly phosphorylated tau increased upon removal of the neurotrophic factor. They showed that staurosporine, a broad-spectrum kinase inhibitor, attenuated

tau phosphorylation and had a dose-dependent protective effect on cell morphology and viability after NGF deprivation in PC12 cells.

Although our results indicate that retraction of neurites during NGF deprivation is not the result of an increase in $[Ca^{2+}]_i$, it could be caused by a reduction in $[Ca^{2+}]_i$.

In summary, we have shown that the retraction of neurites and cell death induced by NGF deprivation of NGF-differentiated PC12 cells are two unrelated processes. First there was a difference in timing: morphological changes already appeared during the first hours of deprivation, whereas cell death occurred after 24–48 h. Therefore, after 24 h of NGF deprivation, the total neurite length is affected more than is cell survival. We can state that cell structure is more sensitive to NGF deprivation. Second, neurite retraction and cell survival were influenced in a different way by the test compounds. Flunarizine and nimodipine protected the NGF-deprived PC12 cells against cell death, whereas BAPTA-AM and EGTA failed to do so. This suggests that NGF deprivation-induced cell death does not result from an influx of extracellular Ca^{2+}. The mechanism by which flunarizine and nimodipine protect against cell death is not known. It is possible that they protect against cell death by acting on the intracellular Ca^{2+} pool or by influencing the permeability of the plasma membrane. None of these compounds prevented the retraction of neurites seen during NGF deprivation. Our observations support the finding that this retraction of neurites is not the result of an increase in $[Ca^{2+}]_i$. The actual role of Ca^{2+} in the survival and morphology of PC12 cells during NGF deprivation needs to be investigated.

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