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Quantitation of the Growth-Associated Protein B-50/GAP-43 and Neurite Outgrowth in PC12 Cells

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A combined assay to measure neurite outgrowth and B-50/GAP-43 levels in PC12 cells is reported. During NGF-induced neuritogenesis, B-50/GAP-43 expression was monitored by enzyme-linked immunosorbent assay (ELISA). Neurite outgrowth was quantified at the same time by the use of video image analysis. Sensitivity and reliability of the methods are shown with a dose–response and time curve of β-NGF-induced neuritogenesis. A linear increase in total length of neurites was induced by concentrations of β-NGF ≥5 ng/ml and was accompanied by a linear increase in the amount of B-50/GAP-43. The combined methods presented here can conveniently and reliably establish subtle changes in neurite outgrowth and intracellular protein contents.

Key words: pheochromocytoma cells, nerve growth factor, video image analysis, cell culture, neurite outgrowth, ELISA

INTRODUCTION

PC12 cells are a clonal line, derived from a rat adrenal pheochromocytoma (Greene and Tischler, 1976). When grown in conventional media, PC12 cells resemble chromaffin cells. The addition of nerve growth factor (NGF) leads to a rapid change of their phenotype and neuronal characteristics are readily observed. These cells therefore serve as a model to study neuronal differentiation by NGF. Neuritogenesis of PC12 cells is accompanied by enhanced expression of the growth-associated protein B-50/GAP-43 at the level of both mRNA and of protein (Karns et al., 1987; Van Hooff et al., 1986). We describe here a method for the combined quantitation in cultures of PC12 cells of intracellular B-50/GAP-43 levels by enzyme-linked immunosorbent assay (ELISA) and average neurite length per cell using automated video imaging.

MATERIALS AND METHODS

Culture Conditions

PC12 cells were grown at 37°C, 7.5% CO_2 in Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal calf serum (Flow, USA), 5% heat-inactivated horse serum (Flow), penicillin (Flow; 100 IU/ml), and streptomycin (Flow; 100 μg/ml). Cells were dislodged from the culture flasks (Nuncleon Delta, Denmark) by repeated pipetting. After centrifugation (1,200 rpm, 5 min) medium was replaced by chemically defined N1 medium (Bottenstein, 1985). To obtain cultures containing separate cells, the cells were dispersed through a 22-gauge needle, plated at low density (1×10^4 cells per well, minimum volume 80 μl) in 96-well microtiter plates (Nunc), previously coated with 80 μl poly-L-lysine 50 μg/ml (Sigma, USA) for 1 hr and allowed to adhere for 1.5 h before addition of β-NGF (Boehringer, FRG). After incubation for the appropriate time, cells were fixed by adding to the cultures 1 vol 4% paraformaldehyde (PFA) in DMEM, for 1 hr at room temperature followed by PFA for an additional hour at room temperature. Cells were permeabilized with methanol, 20 min at −20°C prior to ELISA.

ELISA for B-50/GAP-43

Relative B-50/GAP-43 levels were determined by an ELISA modified from Doherty et al. (1987). Cultures were rinsed with phosphate-buffered saline (PBS) (3 × 5 min) and preincubated with PBS/10% heat-inactivated horse serum (Flow) and incubated overnight with 1:750 diluted primary antibody—affinity-purified rabbit anti-B-50 IgG batch 8613 KC2 (Oestreicher et al., 1983)—in

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PBS/10% horse serum/0.2% Triton X-100 at 37°C. Cultures were washed with PBS pH 8.6 (3 × 5 min) and incubated with 1:1,000 diluted secondary antibody (GAR-PO $F(ab)_2$) in PBS pH 8.6/10% horse serum for 1 hr at 37°C. Cultures were washed with PBS pH 8.6 (3×) and distilled water (2×) and incubated with o-phenylenediamine 200 mg/ml in 0.2 M Na_2HPO_4, 0.1 M citrate buffer, and 0.02% H_2O_2, 20 min at 37°C. The reaction was terminated by the addition of an equal volume of 4.5 M H_2SO_4, and the optical density was read in a SLT EAR 400 FW Easy Reader (SLT Labinstruments, Austria) at 492 nm with reference wavelength 690 nm; readings were corrected with appropriate controls. ELISA conditions were examined and optimized for cell number, antibody dilution, and incubation times. Subsequently, cultures were rinsed and stained with 0.1% Coomassie brilliant blue (CBB) R250 (Serva, FRG) in 25% acetic acid and 10% isopropanol and used for video image analysis (VIA).

Measurement of the Length of Extensions

The length of the extensions was determined by video imaging, using an IBM AT personal computer, a frame grabber (PCVisionplus, Imaging Technology Inc., Woburn, MA), and a command file (written in TIM versions 3.12, DIFA Measuring Systems, Breda, NL, a software toolkit that gives access to a large number of common image-handling procedures, e.g., controlled erosion, window filtering, object separation, controlled dilation, and image summation). CBB-stained cultures were examined with bright field microscopy, using an Olympus-IM inverted microscope and a 20× objective. Images were recorded with a CCD-camera (High Technology Holland, Eindhoven, Netherlands) and transmitted to the frame grabber, which converted the light microscopic image to a digital matrix of 512 × 512 pixels.

The VIA consists of several stages. During stage A, a threshold level of gray shading is established in order to detect cells and processes in the digitized microscopic image (Fig. 1A). A threshold low enough to detect the whole process must be used; otherwise, parts of it will be lost during the establishment of the processes to be measured (stage D). When the threshold is determined and analysis proceeds in stage B (below), the operator can start selecting a new field.

During stage B, the digitized image is inverted and all objects above threshold are displayed in color in the binary image of the frame grabber monitor. The objects are eroded until lines with a width of 1 pixel remain—the so-called skeleton (controlled erosion) (Fig. 1B).

During stage C, cells are counted, and the background of cell debris or particulate matter is eliminated from the image by gray-level erosion (replacing each pixel value by the maximum pixel value found in an adjustable window size around it; nonlinear window filtering). Objects that disappeared by this procedure are not regarded as cells and not counted (object separation). Cell body boundaries are reestablished by restoring the remaining objects through gray-level dilation.

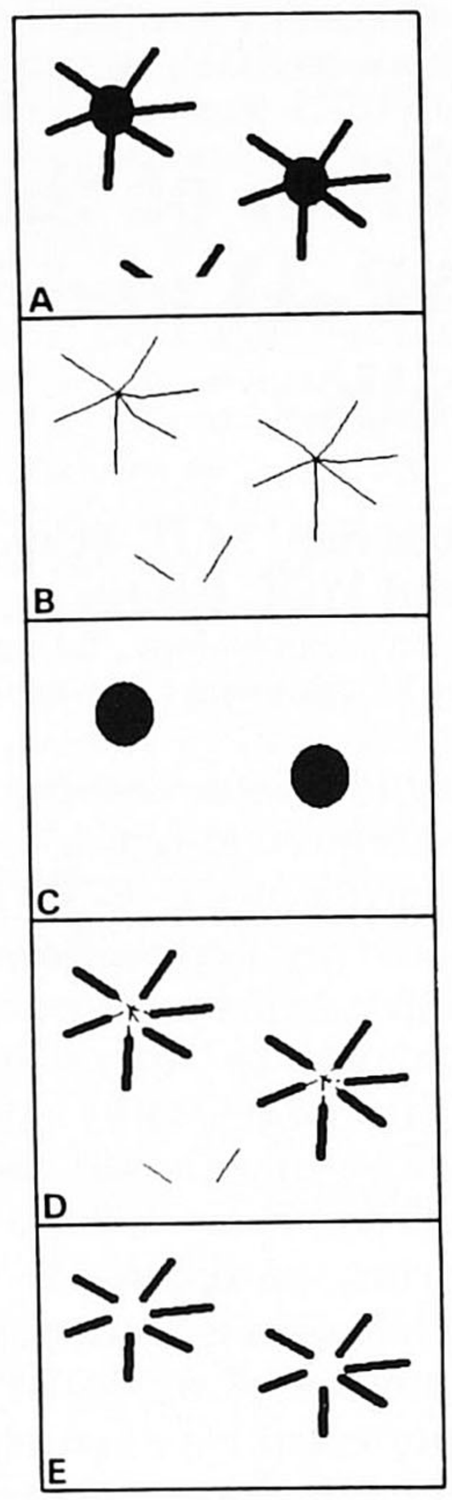

Fig. 1. Schematic diagram of the video image analysis (VIA) procedure. Stage A represents the initial digitized image. After eroding the image, a 1-pixel skeleton remains (stage B). Stage C represents the result of cell body detection and stage D, extension detection. Cell bodies were projected over the skeletons (dashed lines) and processes detected (bold lines). Note that extensions not connected with cell bodies are removed. Stage E shows all extensions finally included in the quantification.

During stage D, the cell bodies detected in stage C are projected over the skeletons of stage B. Extensions are determined by following the remaining parts of the skeleton pixel by pixel, starting from the cell body boundaries (controlled dilation). This stage eliminates processes not connected to a cell body, like processes belonging to cells outside the scope of the field or elongated background structures (scratches).

During stage E, cell bodies are subtracted from the remaining skeletons of stage D and the length is ex-

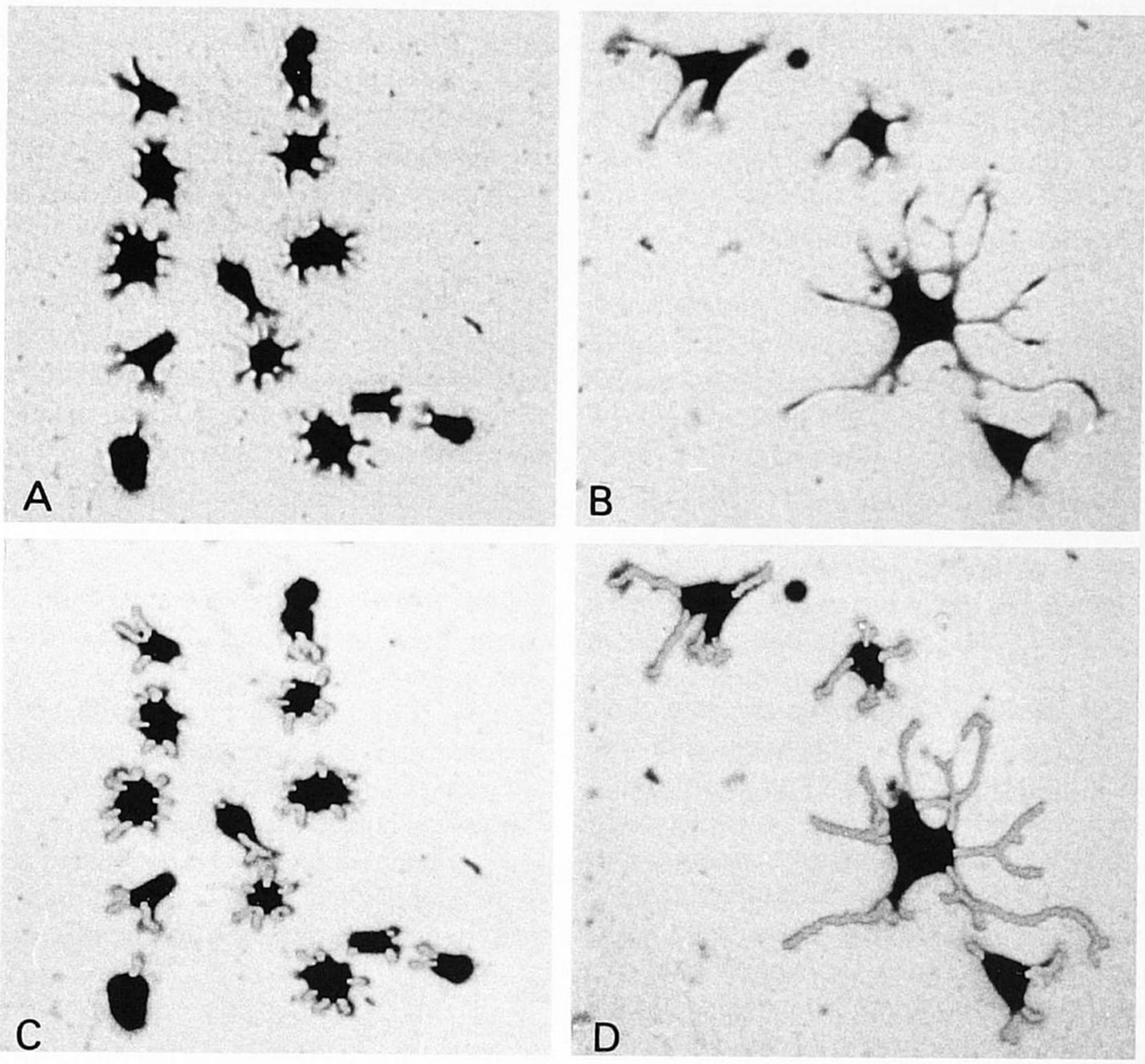

Fig. 2. Neurite formation analyzed by video image analysis (VIA). PC12 cells were treated without (A,C) or with (B,D) 5 ng/ml β-NGF for 24 hr. Digitized images before **(A,B)** or after **(C,D)** VIA. VIA-detected extensions are shown in bitplane overlay (gray; C,D). Photographs were taken directly from the frame grabber monitor.

pressed in pixels per cell. Computer recognized extensions are projected in color over the original digitized microscopic image in order to check the whole processing with the original image (Figs. 1E, 2C,D). Of each well, three fields were analyzed; the mean was taken as the final result of that well.

RESULTS

Time Course of NGF-Induced Neurite Formation

PC12 cells plated on poly-L-lysine-coated microtiter plates were cultured in the presence or absence of 5 ng/ml β-NGF for 24 hr. After fixation, neurite formation was determined. Figure 2 shows the results before (A,B) or after the video-imaging procedure (C,D). Figure 2 shows that nearly all extensions (>98%) were detected by the VIA procedure, both in the presence (C) and in the absence (D) of NGF.

Neurite formation was analyzed after 0, 12, 24, 36, 48, and 72 hr of culture in the presence or absence of β-NGF. Of each well, three fields were analyzed, accounting for 1.5% of the surface of the well. Per time point, 8 wells were assayed, resulting in 750–1,000 analyzed cells. Results are shown in Figure 3.

Cells cultured without NGF showed a significant increase in the length of the processes at 0–12 hr, leveling off at 44 ± 1 pixel/cell at 24 hr. Processes were retracted with increasing time, detected as a decrease in the number of pixels of their processes.

NGF-treated cells showed an almost linear increase in total neurite length ≤ 24 hr, after which time retraction of neurites was detected as a linear decrease in neurite length.

Concentration Dependency of the Effect of NGF

In a separate experiment, PC12 cells were cultured in the presence of 0.0, 0.3, 1.0, 3.0, 5.0, and 9.0 ng/ml β-NGF for 24-hr. B-50/GAP-43 levels and neurite length were measured.

Neurite outgrowth. Using 0.3 ng/ml β-NGF, no

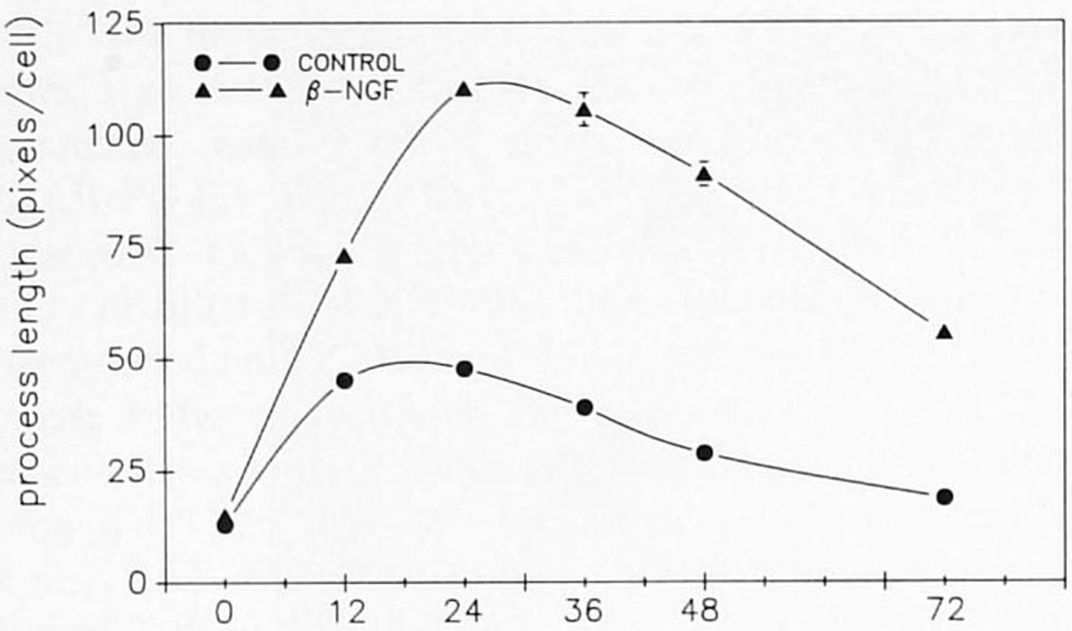

Fig. 3. Time course of process formation of PC12 cells in the presence or absence of β-NGF (5 ng/ml) as quantified by video image analysis (VIA). Each time point represents the mean ±SEM of 8 wells. VIA was performed on 3 fields per well.

significant increase in neurite length was detected compared with untreated cultures. Upon treatment with 1.0, 3.0, 5.0, and 9.0 ng/ml β-NGF, PC12 cells showed a concentration-dependent increase in neurite length reaching 95 ± 2 pixels/cell at the highest concentration, whereas control cultures showed extensions with a length of 48 ± 1 pixel/cell (mean ±SEM). Data from all β-NGF concentrations above zero were significantly different from each other (ANOVA followed by Duncan's multiple range test). Regression analysis showed a semilogarithmic linear relationship between the concentrations of NGF and the length of the extensions relative to control (Fig. 4A, R = 0.99, n = 8).

B-50/GAP-43 levels. With increasing concentrations of β-NGF administered to PC12 cells, increased expression of B-50/GAP-43, a neuronal growth cone marker, is induced (Fig. 4A). Similar to the effect on neurite length, B-50/GAP-43 levels measured by ELISA demonstrate a NGF concentration-dependent linear increase. A semilogarithmic linear relationship was observed between concentrations NGF and B-50/GAP-43 levels (R = 0.99, n = 7). A complete and linear correlation between B-50/GAP-43 levels and neurite outgrowth was found as well (Fig. 4B, R = 0.99, n = 8).

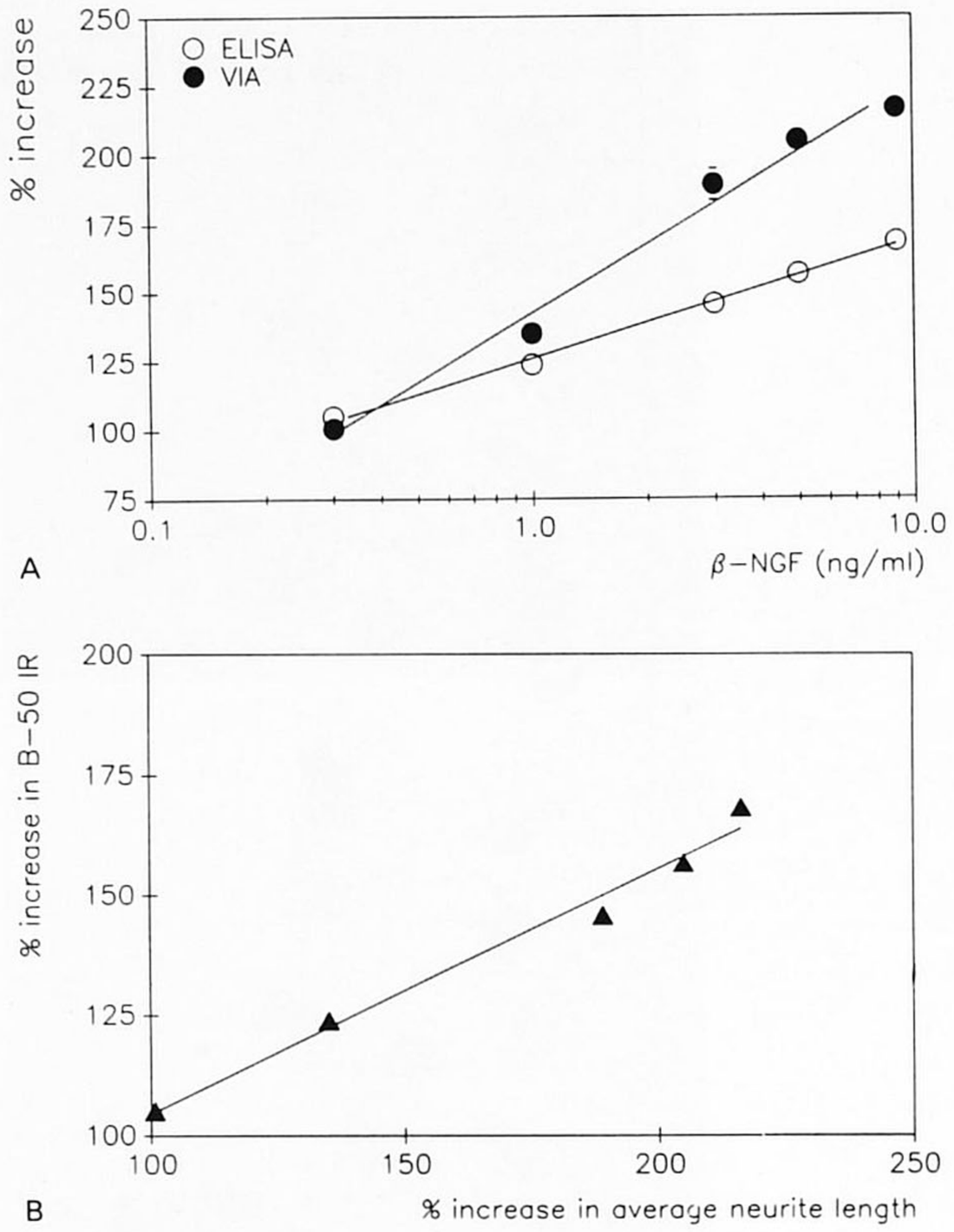

Fig. 4. Concentration dependency of NGF-induced neurite formation and B-50/GAP-43 levels. Neurite length was measured by VIA and expressed as number of pixels per cell. Data were obtained from 8 wells per concentration. Video image analysis (VIA) was performed on 3 fields per well. Regression analysis was executed on the results of 1.0, 3.0, 5.0, and 9.0 ng/ml β-NGF. R = 0.99, n = 8 for VIA and n = 7 for ELISA **(A).** Values of 0.3, 1.0, 3.0, 5.0 and 9.0 ng/ml β-NGF were significantly different from each other both for neurite length and B-50/GAP-43 levels (ANOVA followed by Duncan's multiple range test). Regression analysis of neurite outgrowth vs. B-50/GAP-43 levels **(B).** R = 0.99, n = 7.

DISCUSSION

PC12 cells are frequently used as a model to study neuronal differentiation, morphologically expressed by the formation of neurites. Neurite outgrowth is most commonly measured by taking micrographs of cells and measuring the length of the extensions manually, or by counting the number of neurite-bearing cells, where a neurite is defined as a process with a length of at least the diameter of the cell body (and bearing a growth cone at its tip) (Burstein and Greene, 1978; Skaper et al., 1983; Katoh-Semba et al., 1984; Wagner and D'Amore, 1986; Liesi et al., 1989). Both methods are tedious and less suitable to quantify the dynamics or subtle changes of outgrowth in large numbers of cells. We developed a method to monitor protein expression and neurite outgrowth in the same culture using an ELISA, followed by an image-handling sequence to measure mean neurite length per cell directly from video images of fixed and stained cultures.

The VIA procedure quantifies the total length of processes of both responding and nonresponding cells present in the field that was analyzed. Therefore, it avoids selection of cells; only the threshold setting and

field selection are left to the operator's decision. Correct threshold settings are readily checked, since the detected cells are shown directly as a binary image on the frame grabber monitor and threshold levels can be adjusted in order to correct for cells and extensions below threshold, before continuing the analysis. For all experiments, three fields per well were analyzed for process length to avoid subjectivity by the field selection.

Standard errors ($n = 8$) were small ($\leq 5\%$) and mainly due to (1) variations in threshold settings to correct for changes in light intensity of the images produced by small alterations in lamp current, fluctuations in optic features of the wells, and aberrations in the microscope optics; and (2) variations between the different cultures.

The use of computer-assisted VIA for quantitation of neurite length has been described (Gayer and Schwartz, 1983; Ford-Holevinski et al., 1986). Matsumoto et al. (1990) recently described a video image method to determine neurite outgrowth of cortical neurons. Their method differs from ours in that it requires extensive user input to identify neurons. Furthermore, high demands on stable illumination are made to avoid false positive extensions. The present method was developed to minimize user handling and automatically quantitate neurite formation of irregularly shaped cells using nonlinear window filtering to differentiate between cell bodies and noise.

Certain restrictions that also apply for other methods remain present in the VIA method. Cells must be grown as separated as possible (Gunning et al., 1981), since fields containing clumps of cells cannot be analyzed properly. In those cases, neurites might be obscured. Cultures should be sparse enough to prevent overlapping or parallel growing processes since these cannot be distinguished by VIA and would reduce the average process length. Moreover, contact between neurites prevents further growth of the neurite and may even inhibit elongation of other neurites (Van der Neut et al., 1990).

Direct antigen binding assays in microwells have been shown to be a highly sensitive method to quantify protein expression in cultured cells (Doherty et al., 1984). Indeed we were able to pick up enhancement of B-50/GAP-43 levels as little as 5% in 1×10^4 cells per well, induced by 0.3 ng/ml β-NGF. Results were highly reproducible with standard errors smaller then 5%. Combined with VIA, this allows the study of protein expression in relationship to neurite outgrowth in the same cultures.

VIA enabled us to quantitate the induction of neurite formation as early as 12 hr after NGF addition and to follow outgrowth in time (Fig. 4). Moreover, we were able to quantitate the initial development of small processes by PC12 cells in the absence of NGF, after attachment to the substratum. This phenomenon has been observed before when PC12 cells were cultured in chemically defined N1 medium; these extensions might be used for motion (Keshmirian et al., 1989). A time course study showed that development and retraction of these processes is similar to the course of NGF-induced extensions. NGF-induced extensions, however, grew more rapidly and longer (Fig. 4). This result is different from that observed by Skaper et al. (1983), who reported an increase in neurite-bearing cells until 2 days in culture. This may be due to the differences in quantitation methods used or other variables like the type of NGF used (7S vs. β-NGF), its half-life or the passage number of the PC12 cells.

B-50/GAP-43 ELISA and VIA performed on the same cultures treated with various NGF concentrations revealed that increased neurite outgrowth is accompanied by increased amounts of B-50/GAP-43 immunoreactivity and that both parameters follow the same kinetics. A linear increase of B-50/GAP-43 levels as a function of neurite outgrowth was found (re)establishing that B-50/GAP-43 is a marker for neurite outgrowth. Results obtained by overexpressing GAP-43/B-50/GAP-43 in PC12 cells leading to enhanced neurite outgrowth or by its expression in nonneuronal cells with the induction of filopodia suggest that B-50/GAP-43 might be a mediator of the process of neurite outgrowth. Results obtained by van der Neut et al. (1990), who found that expression of B-50/GAP-43 ceases once neuronal contact has been established, support this thought. Additional studies will be necessary to validate this hypothesis.

Combined assays for protein levels and average neurite length in cultures with dispersed cells have not been described yet. VIA is a rapid and reliable method to quantify neurite formation, sensitive to establish neurite formation in an early stage, and to quantify subtle changes in neurite length as a result of differences in the amount of NGF. Combined with the ELISA for B-50/GAP-43, it seems especially suitable to analyze the effect of substances interfering with or inducing neuronal outgrowth.

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