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Nitric Oxide Underlies the Differentiation of PC12 Cells Induced by Depolarization with High KCl[1]

Hiroyuki Nakagawa, Masaru Yoshida, and Shigeaki Miyamoto[2]

Department of Biochemical Science, Faculty of Computer and Systems, Engineering, Kyushu Institute of Technology, Iizuka, Fukuoka 820–8502

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Nitric oxide (NO) acts as a cytostatic agent to induce neuronal differentiation of PC12 cells after nerve growth factor (NGF) treatment. We newly subcloned PC12K cells that extended neurites after depolarization with high KCl. Here we present evidence that the neuronal differentiation of PC12K cells caused by depolarization with high KCl is mediated by endogenous NO. The outgrowth of neurites was significantly inhibited by 2 mM *N*-nitro-L-arginine methyl ester (L-NMAE), and 10 mM L-NAME was necessary for complete inhibition. The inhibition of NGF-dependent neurite outgrowth by L-NAME was abolished by depolarization of cells with KCl. The expression of neuronal- and endothelial-NO-synthase in PC12K cells was confirmed by immuno-cytochemical and immuno-blotting analyses with the respective monoclonal antibodies. However, the expression of inducible-NO synthase was not observed in PC12K cells cultured with high KCl under the depolarization conditions with 45 mM KCl. We observed the increase of NO in the differentiated PC12K cells using diaminofluorescein, a novel fluorescent indicator for NO.

Key words: L-NAME, neurite outgrowth, NO, NO-synthase, PC12 cells.

NO, a simple and diffusible free radical, is thought to be one of the most noteworthy biological activators (*1*). In the cell cytosol, NO is synthesized by the enzyme NO-synthase (NOS) from L-arginine. Usually there are three types of NOS present in a cell: nNOS, neuronal NOS, eNOS, endothelial NOS, and iNOS, inducible NOS (*2–4*). nNOS and eNOS of constitutive NOS present in almost all cells require an increase of Ca^{2+} ions and calmodulin for their activation (*5*). In the neuronal system, NO contributes to neurotransmitter release, synaptic plasticity, axonal elongation, and cellular apoptosis (*6*). Hess *et al.* demonstrated that exogenous NO caused retraction of neurites in rat dorsal root ganglion cells (*7*). These observations indicate that NO plays an essential role in the construction of neural networks and in the maintenance of neural function (*8, 9*). In PC12 cell lines, established from a rat adrenal pheochromocytoma, NGF treatment induces differentiation into neuronal cells (*10*). Peunova and Enikolopov demonstrated that exogenous and endogenous NO arrested cell division and induced cytostasis (*11*). Solem *et al.* reported that a PC12 cell variant, overexpressing NGF receptor p140 (*trkA*), induced neurite outgrowth after depolarization with KCl and that this molecular process was controlled by a signal pathway mediated by Ca ions (*12*).

Here our aim is to clarify the role of NO as a cytostatic agent to induce neuronal differentiation of PC12 cells caused by depolarization with KCl. We newly isolated a PC12K subclone which induced neurite outgrowth in a depolarization medium with KCl, and investigated the roles of endogenous NO in PC12K cells during the process of neurite outgrowth induced by NGF treatment and by depolarization with KCl. First, we focused on the neurite outgrowth after suppression of NOS activity by an antagonistic inhibitor, L-NAME. The morphological changes which the cultured cells underwent were studied microscopically by calculating the percentage of cells with neurites and by measuring their lengths. Next, at each phase of PC12K cell differentiation, we confirmed the expression of nNOS, eNOS, and iNOS in PC12K cells by immuno-cytochemical and immuno-blotting analyses with the respective monoclonal antibodies. Further, the endogenous NO molecules synthesized in the cytosol were detected in real time with diaminofluorescein (DAF), a novel fluorescent indicator for NO (*13*).

MATERIALS AND METHODS

Materials—Dulbecco's modified eagle medium (DMEM), a 1:1 mixture of DMEM and F-12 nutrient solution (DF), horse serum (HS), and fetal calf serum (FCS) were purchased from GIBCO BRL (Gaithersburg, MD, USA). NGF

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[2] To whom correspondence should be addressed. Tel: +81-948-29-7812, Fax: +81-948-29-7801, E-mail: miya@bse.kyutech.ac.jp

Abbreviations: CaMII, Ca/calmodulin dependent protein kinase type II; DAF, diaminofluorescein; DIC, differential interference microscope component; DMEM, Dulbecco's modified eagle medium; DF, 1:1 mixture of DMEM and F-12; F-12, F-12 nutrient mixture (Ham); FCS, fetal calf serum; HS, horse serum; KN-62, 1-[*N*,*O*-bis-[5-isoquinoline-sulfonyl]-*N*-methyl-L-tyrosyl]-4-phenyl-piperazine; L-NAME, *N*-nitro-L-arginine methyl ester; MAP2, mitogen activated protein kinase type 2; NGF, nerve growth factor; NOS, nitric oxide synthase; PBS, phosphate buffer saline; *trkA*, nerve growth factor receptor p140.

(7S), transferrin (bovine), progesterone, sodium selenite, streptomycin sulfate, penicillin, *N*-nitro-L-arginine (L-NAME), and *N*-nitro-D-arginine (D-NAME) were purchased from Wako Chemical (Osaka). Insulin was purchased from Novo Nordisk Biochem. (Franklinton, NC, USA). An acid solution of collagen was purchased from KOKEN (Tokyo). Monoclonal antibodies to NOSs were purchased from Transduction Laboratories (Lexington, KY, USA). The other chemicals used in this study were of special grade from Wako Chemical. The wild type PC12 cell subclone was a gift from Dr. Masami Takahashi (Mitsubishi Kagaku Institute of Life Science, Machida).

Cell Culture—PC12 cells were usually cultured under a humidified atmosphere of 95% (v/v) air and 5% (v/v) CO_2 at 37°C in a complete medium consisting of DMEM supplemented with 5% (v/v) heat-inactivated horse serum, 5% (v/v) heat-inactivated fetal calf serum, 25 U/ml of penicillin, 25 μg/ml of streptomycin, and 110 mg/liter of sodium pyruvate. Serum-free medium consisted of 12 g/liter of DF, 5 μg/ml of transferrin, 5 μg/ml of insulin, 5 nM progesterone, 20 μM ethanolamine, 100 nM sodium selenite, 25 U/ml of penicillin, 25 μg/ml of streptomycin, 15 mM HEPES (pH 7.2), and 2.44 g/liter of $NaHCO_3$. The passage of cells was carried out every 2 or 3 days. The cells were suspended by pipetting and diluted twice by the addition of fresh medium.

Cell Subcloning—PC12 wild type cells were plated on 96-well plates at a density of ~1 cell/well. The growing cells were transferred to collagen coated 96-well plates at a density of ~10 cells/well and cultured for 24 h. Neurite outgrowth was induced by NGF treatment or depolarization with KCl. The cells that extended neurites on both NGF treatment and depolarization with 45 mM KCl were plated on a 100 mm diameter culture dish. A single colony was isolated using a cloning ring.

Induction of Neurites—Cells were plated on a cover-glass bottom culture dish (Meridian Met Tek Corp., Ashland, MA, USA). In most experiments, the dishes were treated with 0.3% (w/v) collagen in an acetic acid solution (pH 3.0), neutralized with phosphate buffer saline (PBS), and then dried under ultraviolet light prior to plating of the cells. Aggregates of cells that frequently formed during culture were dispersed by treatment with PBS containing 0.25% (w/v) trypsin. The cells were then plated on a dish at a density of 5×10^4/dish. After all the cells had adhered to the collagen-coated plate following culturing for 24 h, the medium was replaced with a serum-free medium. We used a serum-free medium for the following two reasons: (i) In PC12 cells, as reported, neurite outgrowth caused by NGF treatment can be suppressed in the presence of serum, and thus this effect was excluded; and (ii) to remove the possibility that the action of the stimulant may be inhibited by unknown factors present in the serum. The induction of the neurites of PC12 cells by NGF treatment was carried out by culturing in the serum-free medium containing 50 ng/ml NGF. Depolarization was achieved by adding KCl to the serum-free medium, to give various KCl concentrations. To examine the effects of osmolarity, an equivalent amount of choline chloride was added to control cultures, and proliferation and non-differentiation of cells were confirmed.

Inhibition of NOS—L-NAME, an NOS inhibitor, was applied to the cells by adding it to the serum-free medium. The effects of L-NAME on neurite outgrowth were examined by calculating the percentage of cells with neurites and by measuring the length of the neurites each day. The effects of D-NAME on neurite outgrowth were also examined simultaneously in some cases. The time when NGF and KCl were added to the serum-free medium was defined as the first day (0 day). On the 6th day, all cells had extended fairly long neurites, but observation of them was difficult because they overlapped. In this study, the observation of cells and measurements were carried out mainly before the 5th day.

Microscopic Measurements—Cells cultured on cover-glass bottom dishes were observed under a differential interference microscope (DIC), ZEISS Axiovert 100TV (Carl Zeiss Jena GmbH, Jena, Germany), generally at 400 × magnification. The DIC images were obtained using a charge coupled device (CCD) video camera (SONY, SSC-M350). The images were acquired with an ARGAS 10 image processor (Hamamatsu Hotonics, Hamamatsu). Image enhancement and background subtraction were carried out while we monitored the images and were finally recorded on video tape.

The Percentage of Cells with Neurites and Their Length—The length of the neurites was estimated automatically by the use of a contour measuring system on the ARGAS 10. As a standard, the image of a microscale was recorded on the image processor for every measurement. The average length of neurites and the percentage of cells with neurites were calculated for any 100 cells seen on the monitor when the microscope stage was moved in a random fashion. The average length of the neurites was calculated by dividing the total sum of the neurite lengths of 100 cells by the total number of neurites. A neurite was defined as a process that was longer than 10 μm, which was nearly equal to the diameter of a cell soma. The percentage of cells with neurites was estimated as the number that had neurites per any 100 cells. The average values and standard deviation were obtained for more than three experiments.

Immuno-Cytochemical Analysis—For indirect immunofluorescence microscopy, cells on glass cover slips were washed twice with PBS at 37°C, fixed with prewarmed 3% formaldehyde in PBS for 20 min at room temperature, washed for 2 × 5 min with PBS, and then incubated with 0.05% triton X-100 in PBS for 5 min. The Permeabilized cells were washed for 3 × 5 min with PBS, blocked, and then incubated with 1% bovine serum albumin (BSA)–PBS for 20 min. Specimens were then incubated with the first antibodies diluted with 1% BSA-PBS for 1 h or overnight at 4°C, washed for 4 × 5 min with PBS, and then incubated with FITC- or rhodamine-labeled anti–rabbit or anti–mouse IgG goat antibodies diluted with 1% BSA-PBS for 1 h. The rhodamine-phalloidin used for staining actin filaments was diluted with the second antibody solution. The immunostained cells were washed for 6 × 5 min with PBS, fixed with 3% formaldehyde in PBS for 5 min, washed with PBS, and then mounted in 0.1% *p*-phenylenediamine in 50% glycerol and 50 mM Na-CO_3 buffer, pH 8.0. All immunofluorescence observations were carried out under a ZEISS Axiovert 100 TV equipped with a cooled CCD camera, MicroMAX (Princeton Instruments, Trenton, NJ, USA).

DAF Staining—Cells were washed twice with Locke's solution (154 mM NaCl, 10 mM glucose, 2.6 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.75 mM K-PO_4 buffer, pH 7.2),

stained with 10 μM DAF in Locke's solution for 30 min at 37°C, and then washed with Locke's solution. DAF fluorescence was observed using a FITC filter set.

SDS-PAGE and Immunoblotting—PC12 cells were washed twice with PBS, detached using a cell scraper and then suspended in PBS. The cells were sedimented by centrifugation at 800 ×*g* for 10 min at room temperature. The precipitate was suspended in a lysis solution (1% SDS, 1 mM sodium vanadate, 10 mM Tris-HCl, pH 7.4), boiled for 5 min and then centrifuged at 20,000 ×*g* for 20 min at 30°C. The supernatant was mixed with a one-third volume of an electrophoresis sample solution (4% SDS, 20% glycerol, 0.003% bromophenol blue, 4% 2-mercaptoethanol, 0.25 M Tris-HCl, pH 6.8), and then subjected to SDS-PAGE according to the method of Laemmli (*14*), using 10% separating gels. Immunoblotting was performed essentially as described by Towbin *et al.* (*15*). The peptides separated in a gel were electrically transferred to a polyvinylidene difluoride membrane sheet. Alkaline phosphatase–conjugated anti–mouse IgG goat antibodies were used as the second antibodies. Enzyme activity development on the membrane sheet was carried out according to the user's manual for an Alkaline Phosphatase Conjugate Substrate Kit (Bio-Rad Laboratories, Hercules, CA, USA).

RESULTS

Neurite Outgrowth Induced by Depolarization with KCl—NO may be a key molecule that arrests mitogenic activity and induces transition from a proliferative phase to cytostatis in the differentiation of neuronal cells (*11*). We aimed to clarify the roles of NO in the neuronal differentiation of PC12 cells caused by depolarization with KCl. We newly isolated a subclone, PC12K cell from a wild type PC12 cell. PC12K cells, when adhered to a collagen-coated plate, can survive for more than 2 weeks in the presence of high KCl even in a serum-free medium. In contrast, PC12K cells cannot grow on a non-coated plate under the same conditions. Further, for PC12K cells, we found neurite outgrowth was induced by depolarization with KCl in a serum-free medium, but could not detect any morphological differences between the neurites induced by NGF treatment and those induced by depolarization with KCl. In addition, the neurites induced under both conditions formed clear growth cones at the tips (Fig. 1). To clarify a switching mechanism triggered by NO, we examined whether or not inhibition of NOS activity suppresses neurite outgrowth caused by depolarization with KCl in the presence of L-NAME. When cells were depolarized with 45 mM KCl, the percentage of cells with neurites increased with the number of days in culture and reached about 80% after 4 days. On depolarization with 45 mM KCl in the presence of 2 mM L-NAME, the percentage of cells with neurites continued to increase linearly with time to about 50% after 4 days (Figs. 1 and 2A). We examined the induction of neurite outgrowth by KCl depolarization in the concentration range of 10 mM KCl to 80 mM KCl (data not shown). The most effective concentration was about 40 mM KCl, which is close to the concentration previously reported (*12*). In the present study, we carried out depolarization mainly with 45 mM KCl for the induction of neurite outgrowth. The length of neurites increased with the number of days in culture during depolarization with 45 mM KCl, and significantly decreased in the presence of 2 mM L-NAME (Fig. 2B). These inhibitory effects of D-NAME on both variables were not observed in PC12K cells. After treatment of cells with 50 ng/ml NGF in the presence of 2 mM L-NAME, both the percentage of cells with neurites and the neurite length increased for the first 2 days in culture and then decreased with longer culture periods (Fig. 3). In contrast, on depolarization with 45 mM KCl, the NGF-dependent neurite outgrowth increased even in the presence of 2 mM L-NAME. The addition of 2 mM L-NAME alone had no effect on the morphology of the cells.

Depolarization Reduces the L-NAME–Induced Suppression of the Neurite Outgrowth Caused by NG—Next, we studied the effects of depolarization on the NO-triggered neurite outgrowth after NGF treatment. We confirmed this effect of L-NAME on neurite outgrowth by calculating the percentage of cells with neurites and by measuring the length of the processes with days in culture (Fig. 3). The percentage of cells with neurites increased with the culture period to about 25% after 2 days and then decreased in the presence of L-NAME. From 2 to 4 days in culture the rate of increase became almost comparable to that after treatment with NGF alone. The neurite length continued to increase with the number of days in culture after depolar-

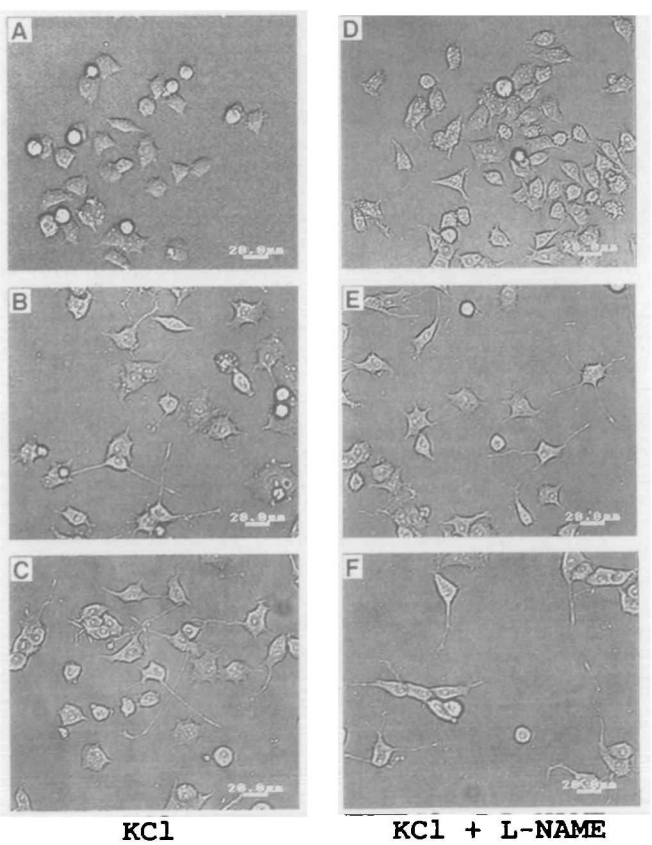

Fig. 1. **Neurite outgrowth of PC12K cells induced by depolarization with 45 mM KCl.** Differential interference micrographs of PC12K cells were taken (A) after 1 day of culture and after adding 45 mM KCl alone, and (D) in the presence of 2 mM L-NAME, (B) after 2 days of culture in the depolarizing medium with 45 mM KCl alone and (E) in the presence of 2 mM L-NAME, and (C) after 4 days of culture in the depolarizing medium with 45 mM KCl alone and (F) in the presence of 2 mM L-NAME. Scale bar, 20 μm.

ization with 45 mM KCl (Fig. 3B). These findings show that depolarization with 45 mM KCl reduced the suppressive action of 2 mM L-NAME, and that neurites extended at the same rate as on treatment with NGF alone (Fig. 3). *N*-nitro-D-arginine methyl ester (D-NAME) did not have inhibitory effects on neurite outgrowth (data not shown). The morphological features of the neurite outgrowth showed that the number of processes decreased gradually with time after NGF treatment in the presence of L-NAME (Fig. 4, A, B, and C). In contrast, the cells depolarized with 45 mM KCl after 2 days extended more neurites with time (Fig. 4D). In the presence of 10 mM L-NAME the percentage of cells with neurites decreased to about 15% after 4 days culture under depolarization conditions, and the average length of the neurites was reduced to a smaller value than 10 μm (Fig. 5).

NO Synthesis by nNOS and eNOS—We studied the expression of nNOS, eNOS, and iNOS in PC12K cells by immuno-cytochemical analysis with anti-nNOS antibodies, anti-eNOS antibodies, and anti-iNOS antibodies labeled with FITC, respectively. PC12K cells cultured in a depolarization medium with 45 mM KCl clearly expressed nNOS

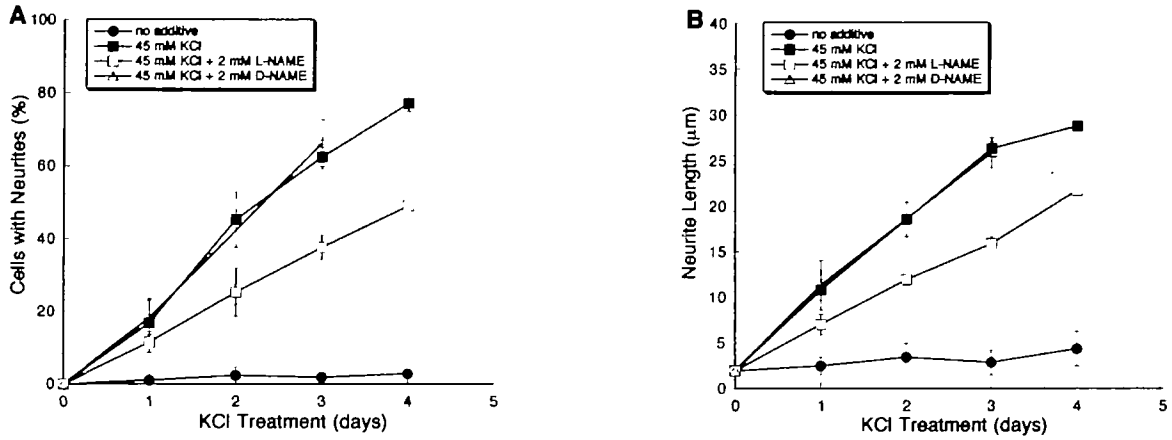

Fig. 2. **A: Percentage of cells with neurites induced by KCl depolarization.** The percentage of cells with neurites was calculated in cultures of PC12K cells in the control medium (●), depolarization medium with 45 mM KCl alone (■), depolarization medium containing 45 mM KCl in the presence of 2 mM L-NAME (□), and depolarization medium with 45 mM KCl in the presence of 2 mM D-NAME (△). Data are means ± SE for 3 to 5 experiments. **B: Length of neurites induced by KCl depolarization.** Neurite length was determined from video micrographs of PC12K cells cultured in the control medium (●), depolarization medium with 45 mM KCl alone (■), depolarization medium with 45 mM KCl in the presence of 2 mM L-NAME (□), and depolarization medium with 45 mM KCl in the presence of 2 mM D-NAME (△). Data are means±SE for 100 neurites for each condition in a single experiment, repeated more than three times.

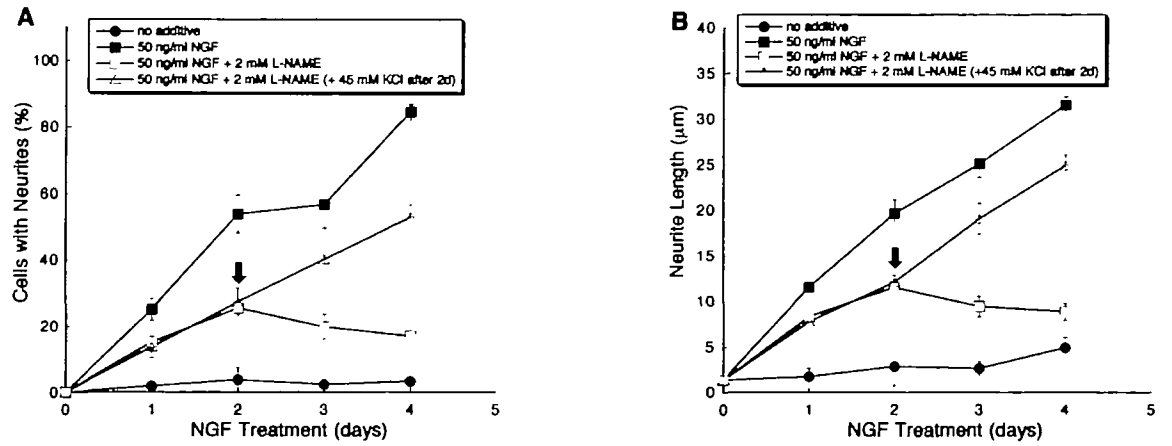

Fig. 3. **A: Percentage of cells with neurites after NGF treatment in the presence of L-NAME.** The percentage of cells with neurites was calculated for PC12K cells cultured for the indicated periods with no additives (●), or treated with 50 ng/ml NGF alone (■), 50 ng/ml NGF in the presence of 2 mM L-NAME (□), or 50 ng/ml NGF in the presence of 2 mM L-NAME and further depolarized with 45 mM KCl after 2 days, as indicated by the arrow (△). Data are means ± SE for 3 to 5 experiments on each day. **B: Length of neurites after NGF treatment in the presence of L-NAME.** Neurite length was determined from video micrographs of PC12K cells cultured for the indicated periods with under no additives (●), or treated with 50 ng/ml NGF alone (■), 50 ng/ml NGF in the presence of 2 mM L-NAME (□), or 50 ng/ml NGF in the presence of 2 mM L-NAME and further depolarized with 45 mM KCl after 2 days, as indicated by the arrow (△). Data are means ± SE for 100 neurites for each condition in a single experiment, repeated more than 3 times.

and eNOS (Fig. 6, A and B). In contrast, only a small amount of iNOS was expressed under the same conditions (Fig. 6C). The expression of nNOS and eNOS was dominant in the cell soma.

Figure 7 shows the results of immunoblotting of PC12K cell extracts with the monoclonal antibodies. The results indicate that the amounts of nNOS and eNOS expressed in the cells were constant independent of NGF treatment and depolarization with KCl. The expression of iNOS was not observed. We also performed immunoblotting with antibodies from other companies, but could not confirm the expression of iNOS. These findings show that the depolarization with KCl does not induce iNOS, different from the results reported by Peunova and Enikolopov (*11*).

Direct observation of NO molecules synthesized in the cytosol became possible with diaminofluorescein (DAF), a novel fluorescent dye for NO (*13*). A small amount of NO was observed uniformly throughout the cytosol of the cells cultured in a complete medium. In contrast, in the cytosol of the cells cultured for 6 days after NGF-treatment or depolarized with KCl, NO molecules were extensively synthesized (Fig. 8). NO synthesis was more active in the central part, especially around the nucleus, than in the peripheral region. These results suggest that NO is a key factor that triggers the switching to cell differentiation.

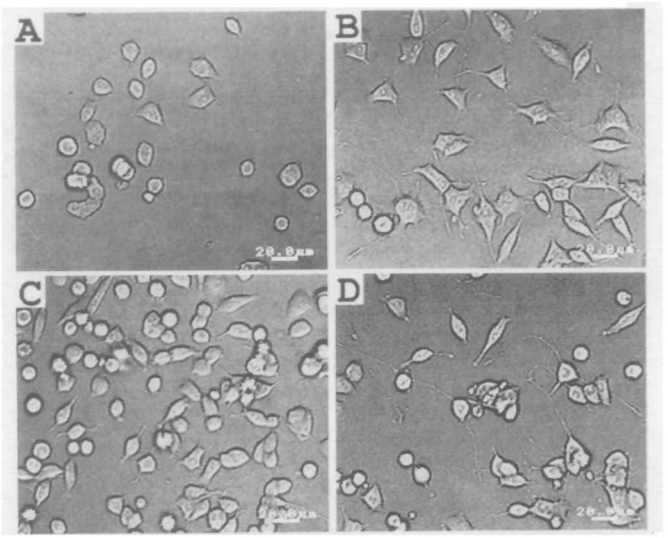

Fig. 4. **Inhibitory effects of L-NAME on neurite outgrowth in PC12K cells after NGF treatment and restoration by depolarization with KCl.** Differential interference micrographs of PC12K were taken (A) before adding NGF, (B) at 2 days of culture after treatment with 50 ng/ml NGF alone, (C) after 4 days of culture with 50 ng/ml NGF treatment in the presence of 2 mM L-NAME and (D) after 4 days of culture with 50 ng/ml NGF treatment in the presence of 2 mM L-NAME and depolarization with 45 mM KCl on the 2nd day. Scale bar, 20 μm.

DISCUSSION

Wild type PC12 cells cultured on a Swiss 3T3 cell layered dish extended neurites after depolarization with KCl (*16*). Solem *et al.* reported that a PC12 cell variant overexpressing *trkA* induced neurite outgrowth after depolarization with KCl in a complete medium (*12*). When cultured in a serum-free medium containing 45 mM KCl, the PC12K cells used in the present experiments continued to grow and extended neurites similar to those that had undergone NGF treatment. The second finding is that the inhibitory effects of L-NAME on neurite outgrowth after depolarization with KCl exhibited a dose dependence different from that of NGF. However, the high concentration of 10 mM L-NAME almost completely inhibited it. This suggests that

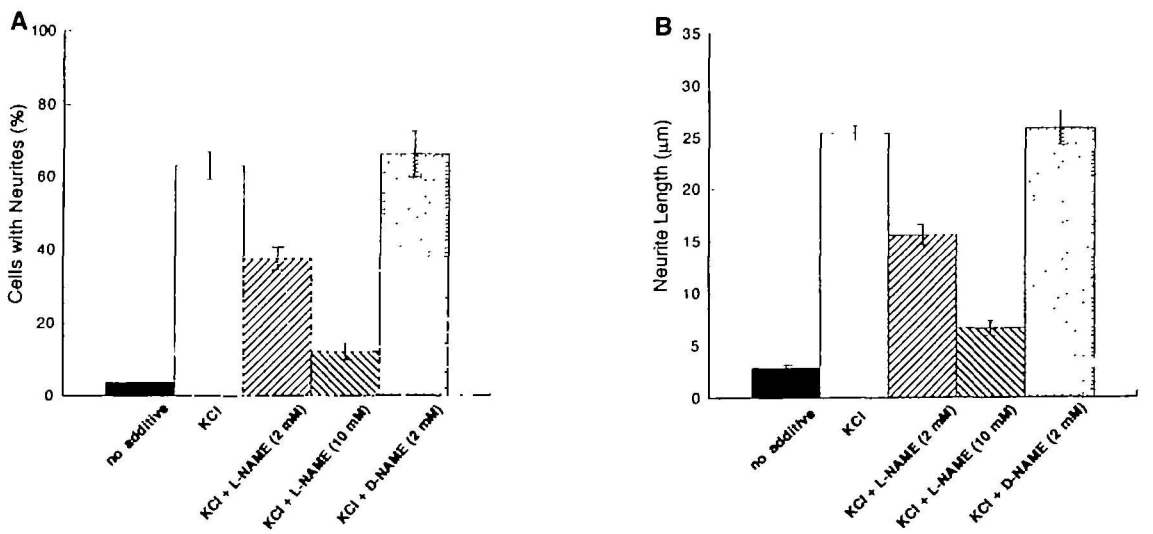

Fig. 5. **A: Dependence of the percentage of cells with neurites on L-NAME and D-NAME.** The percentage of cells with neurites was calculated in cultures in the control medium, the depolarization medium with 45 mM KCl alone, the depolarization medium with 45 mM KCl in the presence of 2 mM L-NAME, the depolarization medium with 45 mM KCl in the presence of 10 mM L-NAME, and the depolarization medium with 45 mM KCl in the presence of 2 mM D-NAME. Data are means±SE for more than 3 experiments after 3 days of culture. **B: Dependence of neurite length on L-NAME and D-NAME.** The dependence of the inhibitory effects of L-NAME and D-NAME on the length of neurites induced by depolarization with 45 mM KCl in the presence of various concentrations of L-NAME and 2 mM D-NAME was examined. Data are means ± SE for more than 3 experiments after 3 days of culture.

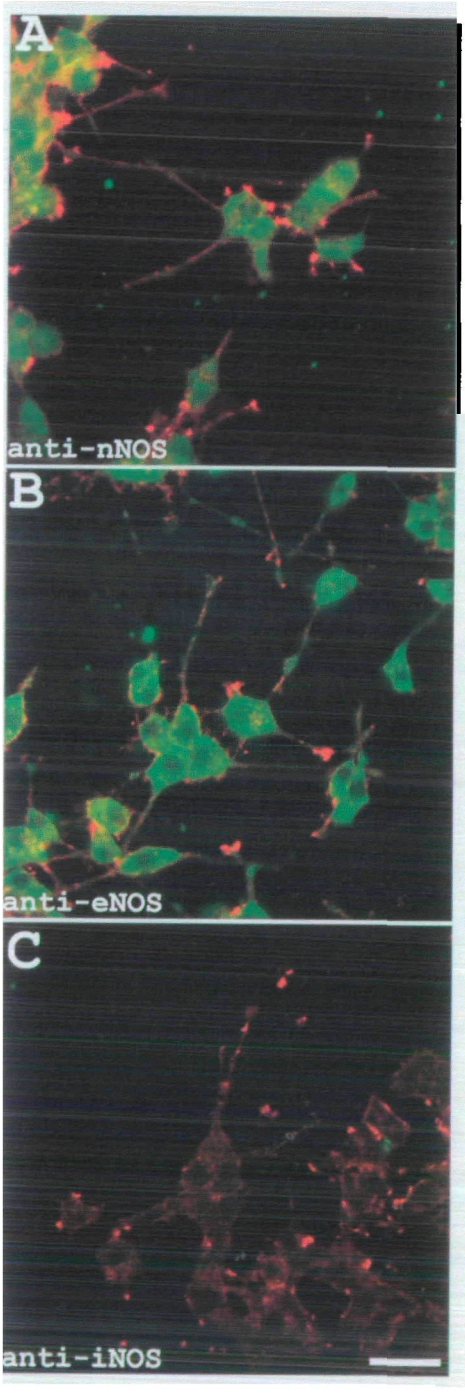

Fig. 6. **Expression of nNOS, eNOS, iNOS, and actin in PC12K cells.** Fluorescent micrographs were taken, with the same exposure, of PC12K cells stained with each anti-NOS monoclonal antibody labeled with FITC, and rhodamine phalloidin, respectively. PC12K cells were cultured for 5 days in the depolarization medium with KCl: (A) stained with anti-nNOS antibodies with rhodamine phalloidin, (B) stained with anti-eNOS antibodies and rhodamine phalloidin, and (C) stained with anti-iNOS antibodies and rhodamine phalloidin. Bar, 10 μm.

the two cases have a common molecular mechanism in a part of the signal pathways involved in neurite formation.

Depolarization with KCl induces Ca ion influx through L-type Ca channels, resulting in an increase of free Ca ions in the cytosol (*17, 18*). This promotes neurite outgrowth, since Ca and calmodulin are essential for the activation of nNOS and eNOS (*5, 18*). This is also consistent with the fact that depolarization with KCl compensates for the inhibitory effects of L-NAME on neurite outgrowth. Some groups have reported the possibility of the involvement of CaMII, Ca/calmodulin dependent protein kinase type II, since a molecular process activated by Ca ions may be important in this process (*12, 20*). That KN-62, a CaMII inhibitor, suppressed neurite outgrowth also indicates the possible involvement of CaMII (*12*). In addition, we consider the following mechanisms to be the molecular pathways related to the activation of CaMII: (i) phosphorylation of CREB (cAMP regulatory element binding protein) with CaMII controls gene transcription, and (ii) phosphorylation of tau 2 and MAP2 (mitogen-activated protein 2) with CaMII inhibits the assembly of microtubules, which have a primary role in cell division. Unfortunately, these mechanisms have not yet been confirmed. On the other hand, Rosen *et al.* reported that an increase in Ca ions induced by depolarization with KCl activated the Ras-MAP kinase pathways (*19–21*).

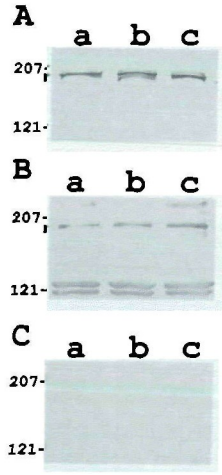

Fig. 7. **Immunoblotting analysis of PC12K cells.** The results on immunoblotting with anti-nNOS antibodies (A), anti-eNOS antibodies (B), and anti-iNOS antibodies (C) after SDS-PAGE for each cell extract are presented: lane a, PC12K cells cultured for 5 days in the complete medium (control); lane b, PC12 K cells cultured for 5 days after NGF treatment; and lane c, PC12K cells cultured for 5 days in the depolarization medium with 45 mM KCl. To each lane the same amount of cell extract, 10 μg protein, was applied. The arrowhead indicates the band of NOS in the cytosol. The numbers show the molecular weights in kDa.

In PC12K cells, the expression of iNOS was not confirmed on either immunocytochemical observation or immunoblotting analysis of cell extracts. The antibodies to each NOS that we used in the present study were obtained from the same company as in Peunova and Enikolopov's study (*11*). They also reported that the expression of iNOS was not observed in PC12-U2 cells, a PC12 cell mutant. In the stage of differentiation induced by depolarization, NO molecules might be synthesized more effectively by Ca-dependent NOS (nNOS and eNOS) than by iNOS. The present results do not contradict the scheme that iNOS is activated at a primary phase, and that nNOS and eNOS mainly work at the later phase.

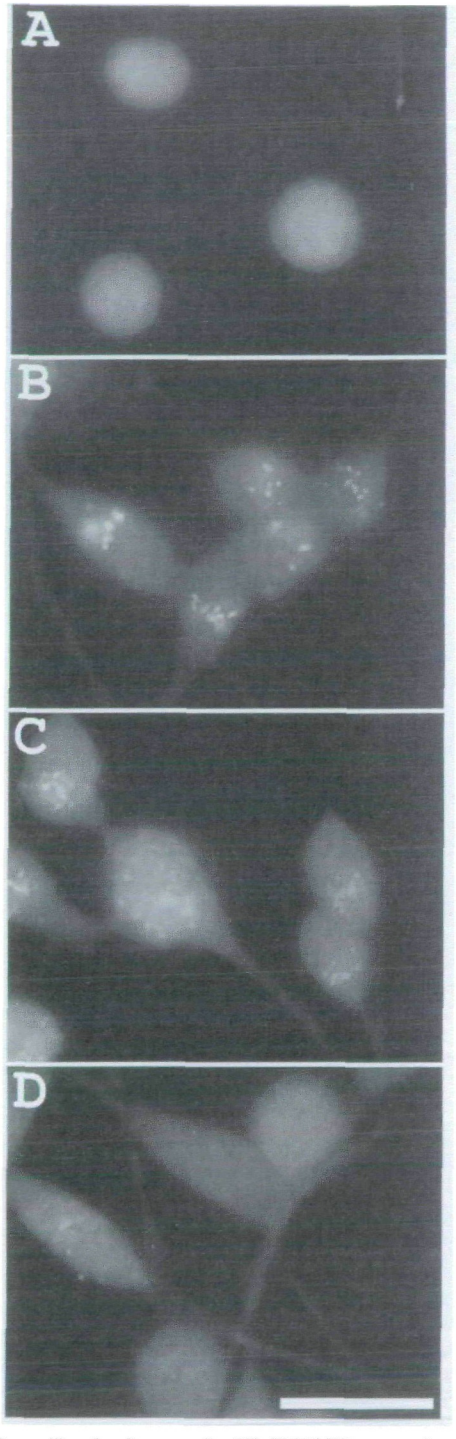

Fig. 8. **NO synthesis observed with DAF.** Micrographs of PC12K cells were taken after staining with diaminofluorescein (DAF) (10 μM), (A) PC12K cells cultured in the complete medium (control), (B) PC12K cells cultured for 6 days after NGF-treatment, (C) PC12K cells cultured for 6 days in the depolarization medium with 45 mM KCl, and (D) PC12K cells cultured for 6 days in the depolarization medium with 45 mM KCl in the presence of 2 mM L-NAME. Bar, 10 μm.

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