Nerve Growth Factor Regulates the Abundance and Distribution of K+ Channels in PC12 Cells

Nutan Sharma, Gabriella D'Arcangelo,* Amy Kleinklaus, Simon Halegoua,* and James S. Trimmer

Departments of Biochemistry and Cell Biology, and *Neurobiology and Behavior, State University of New York at Stony Brook, Stony Brook, New York 11794

Abstract. We examined the effect of nerve growth factor (NGF) treatment on expression of a neuronal delayed rectifier K+ channel subtype, Kv2.1 (drk1), in PC12 cells. Anti–Kv2.1 antibodies recognized a single polypeptide population of M_r = 132 kD in PC12 cell membranes, distinct from the more heterogeneous population found in adult rat brain. In response to NGF treatment, levels of Kv2.1 polypeptide in PC12 membranes increased fourfold. This increase in polypeptide levels could be seen within 12 h, and elevated levels were maintained for at least 6 d of continuous NGF treatment. RNase protection assays indicate that this increase in Kv2.1 protein occurs without an increase in steady state levels of Kv2.1 mRNA following NGF treatment. Immunofluorescent localization of the Kv2.1 polypeptide revealed plasma membrane-associated staining of cell bodies in both untreated and NGF-treated PC12 cells. In undifferentiated cells, intense staining is seen at sites of cell–cell and cell–substratum contact. In differentiated cells the most intense Kv2.1 staining is observed in neuritic growth cones. These studies show that in PC12 cells both the abundance and distribution of the Kv2.1 K+ channel are regulated by NGF, and suggest that PC12 cells provide a model for the selective expression of Kv2.1 in neuritic endings.

OLTAGE-dependent potassium channels, a diverse class of membrane proteins, are fundamental components in the regulation of neuronal excitability (Hille, 1992). Electrophysiological and pharmacological studies have demonstrated the diversity of K+ channels in the mammalian central nervous system, with distinct currents expressed in specific neuronal populations, in distinct developmental periods, or in restricted subcellular (pre- or postsynaptic) sites (Halliwell, 1990). cDNAs encoding a number of K+ channels have been identified over the last three years (reviewed in Salkoff et al., 1992). Probes derived from these cDNAs have been used to demonstrate that K+ channel transcripts have distinct spatial and temporal patterns of expression (Beckh and Pongs, 1990; Swanson et al., 1990; Drewe et al., 1992; Perney et al., 1992; Sheng et al., 1992; Hwang et al., 1992, 1993; Trimmer, 1993). Subtype-specific antibodies have been developed that have been used to localize the expression of the corresponding polypeptides in rat brain. These studies have shown that K+ channel subtypes in rat brain are expressed in specific neuronal populations, and that in these neurons, expression is polarized, in that staining is seen on either axonal or dendritic processes, but not both (Trimmer, 1991; Sheng et al., 1992; Hwang et al., 1993).

One of the most intensely studied of the cloned K+ channels is a member of the *Shab* subfamily of mammalian K+ channels, the rat delayed rectifier Kv2.1 (also known as drk1) (Frech et al., 1989). Kv2.1 mRNA is expressed in rat brain (Drewe et al., 1992), heart (Roberds and Tamkun, 1991) and skeletal muscle (Trimmer, J. S., unpublished data). In the adult rat central nervous system, studies employing antibodies specific for Kv2.1 have shown that Kv2.1 expression is restricted to neurons, where it is present on cell bodies and dendrites but not axons (Trimmer, 1991; Hwang et al., 1993). Multiple Kv2.1 polypeptide isoforms are expressed in rat brain (Trimmer, 1993). The relative proportions of the rat brain Kv2.1 isoforms change during development, implying distinct functional roles for the different forms (Trimmer, 1993). While these results together indicate that the abundance and distribution of the Kv2.1 polypeptide in central neurons is highly regulated, studies addressing the specific cellular mechanisms regulating Kv2.1 expression in rat brain have been hindered by the tremendous cellular heterogeneity of this tissue and the difficulty of approaching these studies in situ.

The clonal cell line PC12 has been a useful model system to study regulation of ion channel expression. In response to the polypeptide neurotrophin NGF, PC12 cells differentiate from immature chromaffinlike cells to cells closely resembling sympathetic neurons. NGF treatment results in the cessation of cell division, the outgrowth of neurites and the capability of generating action potentials (Dichter et al.,

1977; Greene and Tischler, 1982). The development of electrical excitability in these cells is primarily due to an increase in voltage-gated sodium current. This increase is suggested to result from the NGF-induced increase in steady state levels of at least two different types of sodium channel mRNAs (Mandel et al., 1988; D'Arcangelo et al., 1993). Rat brain type II sodium channel mRNA has been shown to increase fourfold after 7 d of NGF treatment (Mandel et al., 1988), while rat peripheral nerve type I (PN1) sodium channel mRNA exhibits a more rapid induction in response to NG treatment, with maximal induction (fivefold) within 5 h of NGF treatment. Undifferentiated PC12 cells have been shown to express at least four different classes of K^+ channels as detected by patch clamp recordings of cell bodies (Hoshi and Aldrich, 1988a,b). Furthermore, neurite growth cones from NGF-treated cells express functional K^+ currents (O'Lague et al., 1985). However the identity of the K^+ channel subtypes identified in molecular cloning studies that contribute to the K^+ currents observed in PC12 cells is not yet known.

Here we report that the Kv2.1 voltage-gated potassium channel gene is expressed in PC12 cells. Treatment of PC12 cells with NGF causes an increase in Kv2.1 polypeptide levels in the absence of a detectable change in Kv2.1 mRNA levels. Immunofluorescent localization of the Kv2.1 polypeptide indicates a preferential localization to points of cell contact in undifferentiated cells and to neuritic growth cones in differentiated cells.

Materials and Methods

Cell Culture

PC12 cells (Greene and Tischler, 1976) were grown in DME (GIBCO-BRL, Gaithersburg, MD) supplemented with 10% horse serum and 5% FCS (both from JRH Biosciences, Lenexa, KS) at 10% CO_2. Cells were maintained on plastic tissue culture dishes and plated on collagen-coated dishes before treatment with NGF. Cells were induced to differentiate using mouse submaxillary gland 7S NGF (Collaborative Research, Inc., Bedford, MA) at 100 ng/ml for 0–6 d. At the time of harvest, each control 100-mm dish had an average of 2.9 × 10^6 cells and each NGF-treated dish had an average of 2.0 × 10^6 cells.

RNA Preparation and Analysis

RNA was prepared and quantified by ultraviolet absorbance at 260 nm and by densitometry of photographs of gels after staining in ethidium bromide as described previously (Trimmer et al., 1990). For RNase protection analysis the 5'-directed Kv2.1 K^+ channel probe pMKP4, corresponding to nucleotides 165–633 (469 bases, encoding amino acids 55–211) of Kv2.1 coding sequence (Frech et al., 1989) and 63 bp of plasmid, was synthesized from the bacteriophage T3 polymerase promoter in Bluescript SK^+. The cyclophilin probe, directed against 107 nucleotides of the coding region, was synthesized from the bacteriophage T7 polymerase promoter in Bluescript SK^+. The probes were synthesized in vitro, as outlined in Ausubel et al. (1990), using α-[^{32}P]UTP. Hybridization and digestion conditions were performed according to the standard protocol provided in the RPAII kit by Ambion, Inc. (Austin, TX). Briefly, both the Kv2.1 and cyclophilin high specific activity antisense cRNA probes were hybridized in the same tube to the sample RNAs (10 μg total RNA) or yeast tRNA (10 μg) for 16 h at 45°C in 80% deionized formamide, 100 mM sodium citrate, pH 6.4, 300 mM sodium acetate, pH 6.4, and 1 mM EDTA. Single stranded RNA was digested with 2.5 U/ml RNase A and 100 U/ml RNase T1 in the supplied buffer. The samples were precipitated with ethanol, subjected to electrophoresis on 5% denaturing polyacrylamide gels, and exposed for autoradiography.

Membrane Preparation

Crude cell membranes were prepared from PC12 cells as follows: cell monolayers were removed from the dish with a pasteur pipette by resuspending them in PBS, pH 7.2, containing 2 mM EDTA, at room temperature. The remaining procedures were performed at 4°C. The cells were pelleted by centrifugation (300 g, 5 min), resuspended in low ionic strength TEP buffer (20 mM Tris-HCl, pH 7.4, and 1 mM EDTA) containing protease inhibitors (2 μg/ml aprotinin, 1 μg/ml leupeptin, 2 μg/ml antipain, 10 μg/ml benzamidine, and 0.2 mM PMSF), and homogenized with 20 strokes of a tight-fitting glass–glass homogenizer. Intact cells and nuclei were removed by centrifugation at 300 g for 7 min. The resultant supernatant was then centrifuged at 50,000 g for 30 min. Membranes were resuspended in TEP buffer and protein content was determined with the bicinchoninic acid method (BCA; Pierce Chem. Co., Rockford, IL) using BSA as the standard.

Antibodies

Antibodies reacting with the Kv2.1 polypeptide were produced and affinity purified essentially as described previously (Trimmer, 1991). Briefly, the pGEX-drk1 antibody was raised against a pGEX (Smith and Johnson, 1988) fusion protein which contains amino acids 506–533 of the Kv2.1 polypeptide. The KC antibody was raised against a synthetic peptide corresponding to the carboxyl terminal, amino acids 837–853, of the Kv2.1 polypeptide (Trimmer, 1991). Both antibodies were affinity purified from crude antisera on a nitrocellulose strip (Olmstead, 1981) containing 1 mg of purified pGEX-drk1 fusion protein (pGEX-drk1 antibody) or pGEX-TC fusion protein (KC antibody). Anti–Thy-1 mAb OX-7 (Mason and Williams, 1980) was provided by Dr. A. Like (University of Massachusetts Medical School, Worcester, MA).

SDS Polyacrylamide Gels and Immunoblotting

Equivalent amounts (30 μg protein) of the crude membrane fractions were size fractionated on 7.5% polyacrylamide gels (Maizel, 1971) and electrophoretically transferred to nitrocellulose membranes (Towbin et al., 1979). Following transfer, nonspecific protein-binding sites were blocked by incubation in Blotto (Johnson et al., 1984), consisting of 5% (wt/vol) nonfat dry milk in TBS, pH 7.5, containing 0.01% (vol/vol) Antifoam A (Sigma Immunochemicals, St. Louis, MO), for 45 min at room temperature. Affinity-purified pGEX-drk1 or KC antibody, diluted 1:50 or 1:100 in Blotto, was incubated with the blocked membranes for 45 min at room temperature. After washes in Blotto, membranes were incubated with affinity-purified horseradish peroxidase conjugated goat anti–rabbit antibodies (Organon Teknika, West Chester, PA) at 1:2,000, visualized using Enhanced Chemiluminescence (ECL) (Amersham Corp., Arlington Heights, IL), and autoradiography on preflashed film. Determination of the apparent M_r of the Kv2.1 polypeptide was made relative to the mobilities of prestained molecular weight standards (Sigma Immunochemicals).

For blocking experiments, affinity-purified pGEX-drk1 antibody diluted at 1:50 was preincubated overnight at 4°C in 1.5 ml of Blotto with 52 μg of either pGEX-drk1 fusion protein, pGEX-TC fusion protein, or pGEX parent protein. Before use, the samples were brought to a final dilution of 1:100 with Blotto and reacted with the blocked membranes as described above.

Indirect Immunofluorescence

All incubations were performed at room temperature. PC12 cells were grown on poly-L-lysine–coated 22-mm^2 coverslips, washed in PBS/1 mM MgCl₂/1 mM CaCl₂, and fixed with freshly prepared 3% (wt/vol) paraformaldehyde in PBS for 10 min at room temperature. After four washes in PBS/1 mM MgCl₂/1 mM CaCl₂ the cells were permeabilized in this wash buffer supplemented with 0.1% Triton X-100 for 5 min. The cells were washed twice in this permeabilization buffer and then nonspecific protein binding sites were blocked by incubation in Blotto, supplemented with 0.1% Triton X-100, for 5 min. The coverslips were washed twice in this blocking buffer and then incubated with affinity-purified pGEX-drk1 antibody, diluted 1:20 in blocking buffer, or the anti–Thy-1 mAb OX-7 hybridoma culture supernatant diluted 1:10 in blocking buffer, for 30 min. After four washes in the blocking buffer, the cells were incubated with affinity-purified rhodamine–conjugated goat anti–mouse (for OX-7) and/or fluorescein-

conjugated goat anti–rabbit IgG (for pGEX-drk1; both from Organon Teknika) at 1:500 for 30 min at room temperature. Each coverslip was mounted on a glass slide in Vectashield mounting medium (Vector Labs., Inc., Burlingame, CA) and the stained cells examined by standard or confocal microscopy. For standard indirect immunofluorescence, double labeled cells were examined on a Zeiss Axiophot microscope under a 63X, 1.25 numerical aperture Zeiss oil immersion lens (Carl Zeiss, Inc., Thornwood, NY). Confocal images were obtained using a laser scanning confocal microscope (MRC 600; Bio Rad Labs., Richmond, CA) with a 40×, 1.3 numerical aperture Nikon glycerin immersion lens. Fluorescein-stained cells were illuminated with the 488-nm line of the Argon laser whereas rhodamine stained cells were illuminated with the 514-nm line of the Argon laser. Optical sections of 0.8 µm were collected and stored for later analysis.

Quantification of Immunoblots and RNase Protection Assays – Linearity of Response

Kodak X-Omat film was preflashed to ensure linearity prior to autoradiography. The relative levels of protein and RNA were determined by densitometry using an ultroscan XL laser densitometer (LKB Instruments, Inc., Bromma, Sweden). The data were analyzed using the Gelscan XL software package (LKB Instruments, Inc.). To determine if immunoblots gave an accurate measure of the relative levels of Kv2.1, increasing amounts of PC12 crude membrane fractions (15–210 µg) were subjected to SDS-PAGE on a 7.5% polyacrylamide gel and then transferred to nitrocellulose. The immunoblots were probed with affinity-purified pGEX-drk1 antibody and the signal was detected using ECL and autoradiography on preflashed film. The signals were then quantified by densitometry. Results indicate that the signal becomes somewhat saturated and nonlinear beyond 150 µg of membrane protein. As we typically analyze 30 µg of membrane protein, we conclude that any calculated fold increases in Kv2.1 polypeptide expression over fivefold may be an underestimate of the true level of increase. To determine if the autoradiograms of RNase protection assays gave an accurate measure of the relative levels of Kv2.1 mRNA, increasing amounts of Kv2.1 cRNA (1–20 µl), transcribed in vitro, were subjected to RNase protection analysis as described above. Alternatively, increasing amounts of adult rat brain RNA (1–10 µg) were added to tubes and analyzed. The signals, which were similar in intensity to that seen in the PC12 RNA samples, increased linearly over a 10-fold range of both the cRNA and brain RNA samples.

Results

Expression of the Kv2.1 Polypeptide in PC12 Cell Membranes

An antibody raised against a fragment of the Kv2.1 polypeptide expressed in bacteria was used in immunoblot assays to define the relative abundance and molecular characteristics of the Kv2.1 polypeptide in PC12 cells. Crude membrane fractions prepared from undifferentiated PC12 cells were compared to similar membrane preparations from adult rat brain. As shown in Fig. 1 A, the Kv2.1 polypeptide in untreated PC12 cells was detected on immunoblots with the affinity-purified polyclonal antibody pGEX-drk1 (Trimmer, 1991). A single, sharp immunoreactive band of M_r = 132 kD was present in the membranes isolated from undifferentiated PC12 cells. This band comigrated with the largest Kv2.1 isoform that was present in the total adult rat brain membrane sample (Fig. 1 A). The fact that the Kv2.1 polypeptide population in membranes isolated from PC12 cells was less heterogeneous than that in whole brain preparations was not surprising given the clonal nature of PC12 cells and the tremendous cellular heterogeneity of the mammalian brain. The levels of Kv2.1 polypeptide present in membranes isolated from undifferentiated PC12 cells were ~10% of the levels of Kv2.1 polypeptide found in membranes prepared in a similar manner from adult rat brain (normalized for membrane protein). This is consistent with our results showing
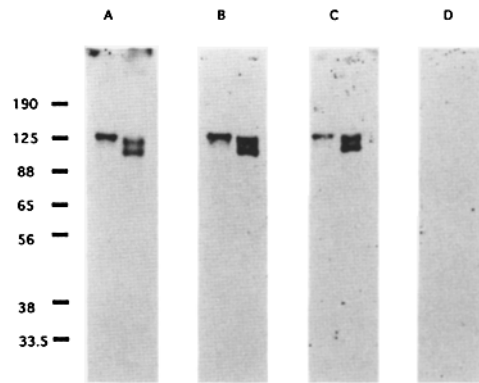

Figure 1. Analysis of specificity of the Kv2.1 antibody immunoreactivity to PC12 membranes. Crude PC12 membranes (30 µg) and crude brain membranes (15 µg) were subjected to fractionation on 7.5% polyacrylamide–SDS gels and electrophoretic transfer as described. Affinity-purified anti–Kv2.1 antibody was preincubated either alone (A), or with equal amounts of the pGEX-2 parent protein (B), the pGEX-TC fusion protein which contains a region of the Kv2.1 polypeptide downstream of the anti–Kv2.1 antibody recognition sequence (C), or the pGEX-drk1 fusion protein recognition sequence (D). The anti-132 kD immunoreactivity is lost upon preincubation of antibody with the pGEX-drk1 fusion protein (D), but is retained upon preincubation with either the parent fusion protein or the heterologous pGEX-KC fusion protein (B and C), indicating that the 132-kD band in PC12 membranes represents endogenous Kv2.1 polypeptide.

the lower levels of Kv2.1 mRNA in total cellular RNA from PC12 cells versus brain (see below).

The specificity of the reaction of the pGEX-drk1 antibody with the M_r = 132-kD PC12 polypeptide was confirmed by blocking experiments. Affinity-purified antibody was preincubated with bacterial fusion proteins containing the fragment of the Kv2.1 polypeptide used to generate the polyclonal antibodies, the parent protein containing no Kv2.1 sequence, or a fusion protein containing a fragment of the Kv2.1 polypeptide outside of the region used as the immunogen. Preincubation of the affinity-purified antibody with the homologous, but not the control or heterologous bacterially
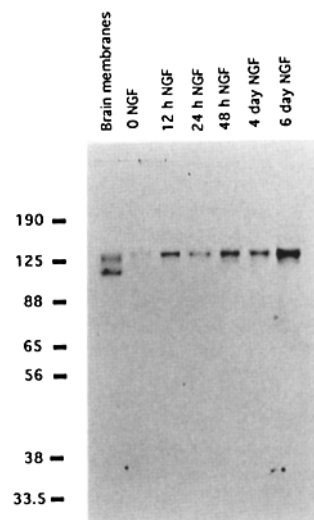

Figure 2. Time course of NGF-induced increase in Kv2.1 polypeptide expression. PC12 cells were cultured on collagen for 6 d. NGF, at a final concentration of 100 ng/ml, was added to the dishes at various times to yield the indicated periods of NGF treatment. Membranes were analyzed via immunoblot, as described previously, and reacted with the affinity-purified pGEX-drk1 antibody. In this experiment, levels of Kv2.1 polypeptide increased (6× over control) by 12-h incubation in NGF, and remained elevated throughout the 6-d time course.

expressed protein resulted in a loss of immunoreactivity to the $M_r = 132$-kD Kv2.1 polypeptide present in these PC12 membranes (Fig. 1, B–D). A similar profile of specific pGEX-drk1 immunoreactivity to rat brain membranes has also been observed (Trimmer, 1991). The specificity of all subsequent assays using the pGEX-drk1 antibody was confirmed by similar blocking experiments.

The Level of Kv2.1 Polypeptide in PC12 Cells Increases with NGF Treatment

In response to NGF, PC12 cells elaborate the properties of sympathetic neurons and develop electrical excitability. To determine if NGF treatment also leads to an increase in Kv2.1 polypeptide levels, membranes were isolated from PC12 cells after various times in culture with NGF (100 ng/ml for 0–6 d). Immunoblots were performed on equal amounts (30 μg) of membrane protein from each time point. Samples were normalized for membrane protein, as opposed to cell number, as NGF induces the synthesis of additional membrane necessary for neurite outgrowth. With long-term NGF treatment this can lead to an increase in the area of surface membrane, as measured by changes in cell capacitance, of four- to fivefold within the first week of NGF treatment (Garber et al., 1989). We typically obtain a similar increase in membrane protein yield per cell during this same period, indicating that total membrane protein increases roughly with membrane area (data not shown). Thus, normalizing Kv2.1 polypeptide levels to total membrane protein would be predicted to approximate the density of Kv2.1 protein in the cell membrane.

As shown in Fig. 2, Kv2.1 polypeptide levels begin to increase after 12 h of NGF treatment. Further incubation in NGF causes an additional increase in Kv2.1 polypeptide levels which remain elevated for up to 144 h of NGF treatment (4.7 ± 0.7-fold over control, $n = 4$). The slight decline in Kv2.1 polypeptide levels at 24 h of NGF treatment in Fig. 2 is indicative of the variability observed in numerous independent time course experiments. However, Kv2.1 polypeptide levels never failed to increase in any experiment ($n = 25$) upon treatment of cells with NGF. In a separate NGF time course, a similar increase in Kv2.1 polypeptide levels is seen when immunoblots were probed with a second anti–Kv2.1 antibody (KC) (Trimmer, 1991), which recognizes the carboxyl terminal 18 amino acids of the Kv2.1 polypeptide (Fig. 3). As shown in Fig. 3, levels of Kv2.1 polypeptide increased (5.5-fold over control) after 24 h in NGF and remained high at 36 h (5.9-fold over control) and 48 h (3.8-fold over control) of NGF treatment. Results similar to those obtained with the pGEX-drk1 antibody and presented in Fig. 2 were obtained when the KC antibody was used to analyze membranes from PC12 cells treated with NGF for longer periods (data not shown). Thus immunoblots probed with two distinct antibodies that recognize different regions of the Kv2.1 polypeptide demonstrate comparable increases in Kv2.1 polypeptide levels in response to NGF. The average increase in Kv2.1 polypeptide levels in PC12 membranes upon NGF treatment was determined in a number of independent experiments using immunoblots and densitometry; these data are summarized in Fig. 4. Increases of roughly the same magnitude are observed when immunoblots are performed on samples normalized for total
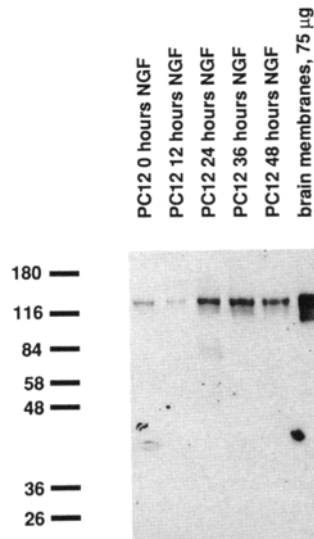

Figure 3. Time course of NGF-induced increase in Kv2.1 polypeptide expression. PC12 cells were cultured on collagen for 2 d. NGF, at a final concentration of 100 ng/ml, was added to the dishes at various times to yield the indicated periods of NGF treatment. Membranes were analyzed via immunoblot, as described previously, and reacted with the affinity-purified KC antibody. In this experiment, levels of Kv2.1 polypeptide increased by 24 h incubation in NGF (5.5× over control) and remained high at 36 h (5.9×) and 48 h (3.8×).

cellular protein (Sharma, N., and J. Halegoua, unpublished data).

We have also determined the NGF-induced increase in Kv2.1 polypeptide levels on a per cell basis. For determining the extent of increase on a per cell basis, NGF-treated PC12 cells were counted upon harvesting, and from these pools of known cell number a crude cell membrane fraction was prepared. When the level of Kv2.1 polypeptide increase per cell is compared to the level of increase normalized to membrane protein, similar values are obtained at early time points (12–24 h; Table I), times at which the NGF-induced increases in membrane area per cell have not yet occurred (Garber et al., 1989). At later time points (2–4 d of NGF treatment), much larger increases (20–30-fold) in Kv2.1 polypeptide levels are obtained when the values are expressed on a per cell basis, than when normalized for membrane protein (four- to fivefold; Table I). The 5–10-fold difference at later time points corresponds roughly in timing and magnitude to the NGF-induced increase in membrane area as measured by capacitance (Garber et al., 1989).

NGF Does Not Up-regulate Levels of Kv2.1 mRNA in PC12 Cells

To determine if the NGF-induced increase in Kv2.1 polypeptide levels was dependent upon an increase in Kv2.1 mRNA levels, we measured Kv2.1 mRNA levels using RNase protection assays. The KV2.1 cRNA probe and the cyclophilin cRNA probe were hybridized in the same tube with total
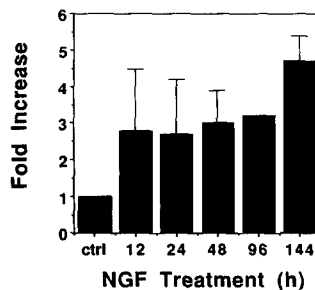

Figure 4. Quantification of NGF induction of the Kv2.1 polypeptide. PC12 cells were treated with NGF for the indicated periods of time. Values are represented as fold-increase versus control cells. 12 h NGF: 2.8 ± 1.7, $n = 6$. 24 h NGF: 2.7 ± 1.5, $n = 7$. 48 h NGF: 3.0 ± 0.9, $n = 11$. 4 d NGF: 3.2, $n = 2$. 6 d NGF: 4.7 ± 0.7, $n = 4$.

Table I. NGF-induced Increases in Kv2.1 Polypeptide Levels in PC12 Cells and Membranes

NGF treatment	Fold increase per cell*	Fold increase per microgram membrane protein‡	Ratio cell/membrane
h			
12	2.5	3	0.83
24	9	4.9	1.8
48	15	3.2	4.7
96	30	3.2	9.4

* Fold increase over control determined by densitometry of immunoblots containing membranes isolated from 10^6 PC12 cells.
‡ Fold increase over control determined by densitometry of immunoblots on the same membranes but normalized for 30 µg of membrane protein.

RNA isolated from control (untreated) PC12 cells and from NGF-treated PC12 cells. The full-length Kv2.1 probe, including vector sequences, was 532 bases, whereas the region corresponding to the Kv2.1 coding region was 469 bases in length. To ensure that the RNase digestion was complete, the size of the undigested probe was verified by gel electrophoresis. In the absence of RNase, the probe appeared at its predicted size of 532 bases (data not shown). In contrast, when the probe was hybridized to total cellular RNA from the brain, which expresses relatively high levels of Kv2.1 mRNA, and then subjected to RNase digestion and electrophoresis, a protected fragment appeared at 469 bp. The cyclophilin cRNA probe, whose levels do not change with NGF treatment (Danielson et al., 1988), was also hybridized with the same samples in which Kv2.1 mRNA levels were measured (Fig. 5). The relative intensity of the protected cyclophilin fragment was monitored in all samples, to ensure that equal amounts of RNA were added in each lane.
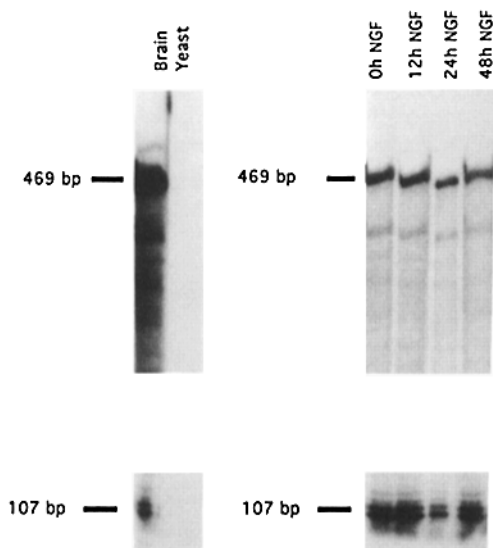

Figure 5. Detection of Kv2.1 transcripts. Results of RNase protection assay performed with RNA from adult rat brain and yeast tRNA and RNA isolated from PC12 cells that were treated with NGF for the indicated periods of time. Upper panels correspond to the Kv2.1 probe, lower panels to the cyclophilin probe. Number refer to the size of the protected fragments.
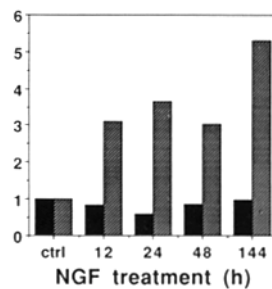

Figure 6. Quantification of RNAse protection and immunoblot results by densitometry. These results are for those cells in which both Kv2.1 mRNA and Kv2.1 polypeptide levels were measured. In order to compensate for small differences in the amount of RNA loaded in each lane, the ratio of the intensity of the Kv2.1 band to the cyclophilin band is represented in the solid bar. The cross-hatched bar represents changes in Kv2.1 polypeptide levels. Values are represented as fold-increase versus control cells. The average fold-change in Kv2.1 mRNA levels is: 12 h NGF: 0.84 ± 0.2, $n = 5$; 24 h NGF: 0.6 ± 0.4, $n = 5$; 48 h NGF: 0.87 ± 0.5, $n = 7$; 144 h NGF: 0.99, $n = 2$. The average fold-increase in Kv2.1 polypeptide levels is: 12 h NGF: 3.1 ± 1.4, $n = 4$; 24 h NGF: 3.7 ± 1.2, $n = 4$; 48 h NGF: 3.0 ± 0.6, $n = 6$; 144 h NGF: 5.3, $n = 2$.

Levels of Kv2.1 mRNA in undifferentiated PC12 cells were found to be substantially lower than those present in an equivalent amount of RNA isolated from adult rat brain, the original source of the Kv2.1 cDNA (Fig. 5). The steady state level of Kv2.1 mRNA in PC12 cells does not increase after 2 d of NGF treatment (Fig. 5), despite an increase in Kv2.1 polypeptide levels over the same time period (see preceding section). Consistent with previous reports (Danielson et al., 1988), steady state levels of cyclophilin mRNA in these samples did not change, indicating that there was not a variation in sample preparation or application.

To ensure that the increase in Kv2.1 polypeptide levels could in fact occur in the absence of increases in Kv2.1 mRNA, both Kv2.1 polypeptide and mRNA levels were determined from the same NGF-treated PC12 cell cultures. PC12 cells were treated with NGF (100 ng/ml) for 0–6 d, and at each time point cells were harvested, pooled by centrifugation, and then split into two fractions. Total cellular RNA was prepared from one fraction, and crude cellular membranes were prepared from the other fraction. Kv2.1 mRNA levels were measured by RNase protection assay, and Kv2.1 polypeptide levels were measured by immunoblot analysis. In Fig. 6, the results of these RNase protection assays are displayed as the ratio of the intensity of the Kv2.1 protected fragment to the intensity of the cyclophilin-protected fragment. This figure also displays the Kv2.1 polypeptide levels in the same cultures for which Kv2.1 mRNA measurements were made. The data presented in Fig. 6 are from only those cultures in which parallel measurements of both Kv2.1 RNA and polypeptide levels were performed. These data taken together show that the NGF-induced increase in Kv2.1 polypeptide levels in these PC12 cell cultures can occur in the absence of an increase in Kv2.1 mRNA levels.

The Kv2.1 Polypeptide Is Enriched in the Growth Cones of NGF-treated PC12 Cells

Indirect immunofluorescence was performed to determine the subcellular distribution of the Kv2.1 polypeptide in undifferentiated and differentiated PC12 cells. Cells were permeabilized prior to staining to allow for access of antibody to cytoplasmically located epitopes. Images were ob-
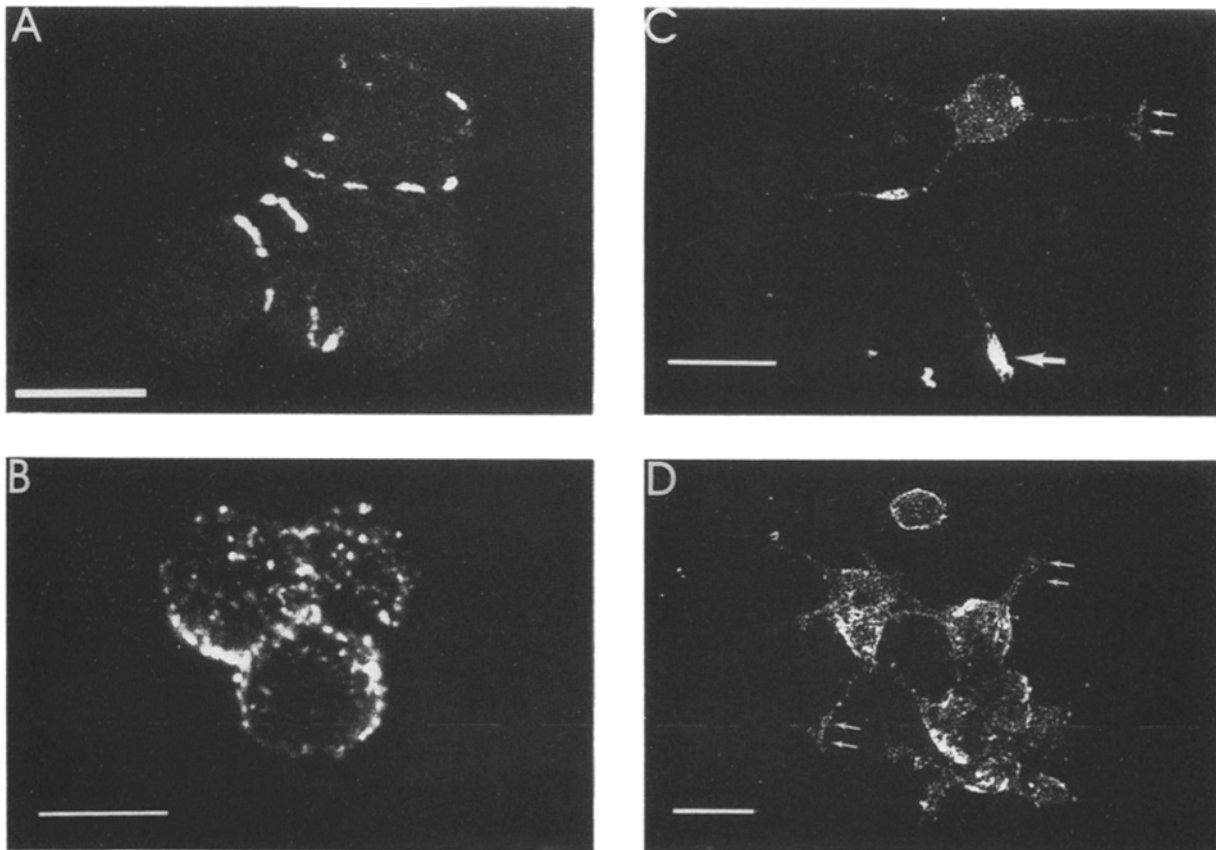
Figure 7. Confocal micrographs of PC12 cells. PC12 cells were grown on poly-L-lysine coated coverslips for 9 d in the absence (*A* and *B*) or presence (*C* and *D*) of NGF. Cells were fixed, permeabilized and stained with either the affinity-purified Kv2.1 antibody (rabbit, *A* and *C*) or the OX-7 mAb (mouse anti–Thy-1, *B* and *D*). Bound antibody was visualized with either fluorescein goat anti–rabbit or rhodamine goat anti–mouse antibodies. *Arrows* indicate growth cones. Shown are projections composed from a series of confocal planes with an optical section size of 0.8 μm. *A*; projection of 27 confocal planes. *B*; projection of 3 confocal planes. *C*; projection of 21 confocal planes. *D*; projection of 31 confocal planes. Bars: (*A* and *B*) 10 μm; (*C*) 50 μm; (*D*) 25 μm.

tained by confocal microscopy. Shown are projections composed from a series of confocal planes with an optical section size of 0.8 μm (see Fig. 7 legend for additional details). The projections are composed of the number of confocal planes necessary to view the entire thickness of the cell and neurite, such that the resultant image is representative of the image viewed by sectioning through the entire cell. In undifferentiated PC12 cells Kv2.1 staining was found at regions of cell–cell and cell–substrate contact (Fig. 7 *A*). A different pattern of staining was observed with a mouse mAb (OX-7) specific for Thy-1, a cell surface glycoprotein previously shown to be present in the plasma membrane of PC12 cells (Richter-Landsberg et al., 1985). Anti-Thy-1 staining was punctate and distributed throughout the periphery of the cell membrane, regardless of cell–cell contact (Fig. 7 *B*). The distribution of the Kv2.1 K$^+$ channel in undifferentiated PC12 cells is clearly unique in its restricted localization among the PC12 membrane proteins assayed.

After 9 d of NGF treatment, which results in up-regulation of both Kv2.1 and Thy-1 (Richter-Landsberg et al., 1985) polypeptides, prominent staining with both the Kv2.1 and Thy-1 antibodies was maintained in the cell body (Fig. 7, *C* and *D*). The Kv2.1 antibody no longer exhibited localized staining at regions of cell–cell or cell–substrate contact; punctate staining was seen over the entire surface of the cell

body (Fig. 7 *C*). The Kv2.1 antibody also exhibited intense staining of the growth cone region present at the distal tips of certain neuronal processes whose growth is induced by NGF (Fig. 7 *C, large arrow*). Not all growth cones exhibited intense staining, in many the staining was only comparable to that seen in the cell body and neurite shaft (Fig. 7 *C, small arrows*). Staining with the anti-Thy-1 mAb was punctate but uniformly distributed over the entire cell body and neuritic membrane, including all of the observed growth cones (Fig. 7 *D, arrows*). No intense growth cone staining was observed with the anti-Thy-1 antibody.

To more quantitatively determine the distribution of Kv2.1 and Thy-1 in the growth cones of differentiated PC12 cells, experiments were performed in which the same cultures were double labeled with the Kv2.1 and Thy-1 antibodies. In those cells that extended neurites (91% of all cells), 79 of the 113 observed growth cones (69.9%) exhibited intense, localized Kv2.1 staining similar to the growth cone labeled by the large arrow in Figure 7 *C*. The remaining growth cones exhibited a level of labeling comparable to that observed on the cell body and neuritic shaft, as in the growth cones labeled with the small arrows in Fig. 7 *C*. In all cells examined Thy-1 staining was found at comparable levels throughout the cell body and neurite. These results indicate that the marked difference in intensity between the cell body and growth cone

Kv2.1 staining does not result from differences in geometry between these different subcellular domains or other non-specific effects.

Discussion

Kv2.1, a rat brain delayed rectifier K$^+$ channel, is expressed in PC12 cells and its expression is regulated by NGF. Kv2.1 is the first K$^+$ channel gene shown to be expressed in PC12 cells. Increases in Kv2.1 polypeptide levels are first observed within 24 h of NGF treatment, and remain elevated for up to 144 h of treatment. This is the first evidence that specific K$^+$ channels in PC12 cells are regulated by NGF. NGF does not, however, induce an increase in steady state levels of Kv2.1 mRNA in the same cells which express elevated levels of Kv2.1 polypeptide, implying that mechanisms other than regulation of gene expression can affect ion channel expression in PC12 cells.

NGF has been shown to induce the expression of other proteins in a transcription-independent manner. Polypeptide levels of NILE[1] (NGF-induced large external glycoprotein) increase in response to NGF (McGuire et al., 1978). Steady state levels of NILE mRNA are not elevated in response to NGF (Sajovic et al., 1987). The NGF-induced increase in neurofilament H polypeptide (NF-H) also appears to be transcription independent (Lindenbaum et al., 1988). NF-H polypeptide levels rise with 8–14 d of NGF treatment, in the absence of increased NF-H mRNA levels. Thus, the NGF-induced increase in both NILE and NH-H polypeptide levels occurs via translational and/or posttranslational mechanisms. However, quantification of the NGF-induced increases in NILE and NF-H polypeptides was not performed. Recently, Frederickson et al. (1992) demonstrated that NGF treatment results in the rapid (within 30 min of NGF addition) phosphorylation of the translation initiation factor eIF-4E. Enhanced phosphorylation of eIF-4E is directly correlated with increased activity, which may lead to the modest general increase in translation rates seen upon NGF treatment of PC12 cells (Garrels and Schubert, 1979; Huff et al., 1981). However, it is possible that regulation of eIF-4E or other components of the translational machinery is also important in the more dramatic change in translation of a subset of PC12 cell proteins upon NGF treatment (Greene and Tischler, 1976; Garrels and Schubert, 1979). In differentiating PC12 cells these may include mRNAs involved in the expression of the neuronal phenotype, such as NF-H and Kv2.1. Posttranslational modifications affecting Kv2.1 protein stability, such as changes in the phosphorylation state of KV2.1, could also result in an increase in KV2.1 polypeptide levels. Further analysis of the dynamic aspects of Kv2.1 protein regulation in these cells may shed light on novel mechanisms whereby growth factors, such as NGF, regulate the levels of channel proteins important in conferring electrical excitability to neurons and other excitable cells. NGF regulation of channel proteins appears to involve several different mechanisms. NGF regulation of the Kv2.1 channel protein occurs at the translational and/or posttranslational levels, whereas NGF regulation of the type II sodium chan-

nel correlates with an increase in mRNA levels (Mandel et al., 1988).

Immunofluorescent staining of undifferentiated PC12 cells with the anti-Kv2.1 antibody revealed punctate plasma membrane-associated staining of the cell body, somewhat similar to what is seen in rat central neurons where the somal plasma membrane is intensely stained with antibodies directed against Kv2.1 (Trimmer, 1991). In addition, Kv2.1 appears to be selectively enriched at regions of cell–cell and cell–substrate contact (Fig. 7 A). Staining of undifferentiated PC12 cells with an anti-Thy-1 antibody yields intense staining at the cell periphery, as is typical for any evenly distributed plasma membrane protein, with no detectable enrichment at points of cell–cell or cell–substratum contact (Fig. 7 B). It should be noted that as the PC12 cells used in all of these experiments were permeabilized to allow for Kv2.1 antibody accessibility, the observed staining represents not only surface channels but also any intracellular pools. Future development of antibodies specific for external domains of Kv2.1 will ultimately allow for a more specific analysis of surface Kv2.1 channels.

Immunofluorescent staining of NGF-treated PC12 cells revealed additional and more intense staining of Kv2.1 in growth cones, a pattern not seen for another prominent PC12 membrane protein induced by NGF treatment, Thy-1. In addition to the targeting of Kv2.1 to neurites, NGF treatment also resulted in the redistribution of Kv2.1 staining in the cell body. After 9 d of NGF treatment, Kv2.1 staining is visible throughout the cell body such that there is no longer any localized Kv2.1 staining at regions of cell–cell and cell–substrate contact. The Thy-1 glycoprotein, like Kv2.1, is up-regulated by NGF (Richter-Landsberg et al., 1985), but is apparently targeted in differentiated PC12 cells in a manner distinct from the Kv2.1 polypeptide, as it does not preferentially accumulate in growth cones. It is interesting that in undifferentiated PC12 cells Kv2.1 is targeted and retained at points of cell contact, and in differentiated PC12 cells Kv2.1 is found primarily in the growth cones of developing neurites. In both cases Kv2.1 is present in regions rich in focal adhesions. These are the first data to indicate that the distribution of K$^+$ channels is regulated and may be influenced by interaction with the cytoskeleton. This system can be used as a model for future studies on the cellular mechanisms by which cell surface proteins are targeted in neuronal cells.

Further studies examining the distribution of various cytoskeletal elements involved in focal contacts and cell–cell adhesion junctions may shed light on the way in which Kv2.1 is targeted to specific regions of the cell membrane. Vinculin has been shown to localize to sites of cell–substrate focal adhesion plaques in PC12 cells (Halegoua, 1987). In response to NGF, both focal adhesion plaques and vinculin foci are redistributed to the proximal portion of the neurite and to the growth cone (Halegoua, 1987). The redistribution of the Kv2.1 polypeptide in response to NGF may follow the redistribution of cytoskeletal focal adhesion plaque proteins such as vinculin. Such associations have been described for other ion channel proteins such as the nicotinic acetylcholine receptor (Froehner, 1991). The availability of this dynamic cell culture system displaying induced targeting will facilitate future studies aimed at identifying regions of the Kv2.1 protein important in interactions with the cellular machinery involved in targeting.

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1. *Abbreviations used in this paper*: NF-H, neurofilament H polypeptide; NILE, NGF-induced large external glycoprotein.

NGF has been shown to induce the expression of other proteins that are involved in the development of the PC12 neuronal phenotype (reviewed in Halegoua et al., 1991), including both sodium and calcium channels. Mandel et al. (1988) and D'Arcangelo et al. (1993) have demonstrated that differentiated PC12 cells have elevated levels of both type II and PN1 sodium channel mRNAs; this expression parallels an increase in the density of sodium channels as measured electrophysiologically. NGF also up-regulates the expression of voltage-dependent Ca^{2+} channels in PC12 cells. NGF-treated PC12 cells display an increase in both T type, low threshold Ca^{2+} current (Garber et al., 1989) and N type, high threshold Ca^{2+} current (Plummer et al., 1989). Thus NGF treatment results in the up-regulation of several ion channels known to be involved in maintaining cell excitability, a hallmark of the neuronal phenotype.

In detailed patch clamp analyses of the K^+ channels present on cell bodies of undifferentiated PC12 cells, Hoshi and Aldrich (1988a,b) have demonstrated the existence of at least four distinct voltage-dependent K^+ channels. As Kv2.1 protein is present on the cell bodies of both undifferentiated and NGF-treated PC12 cells, it would be anticipated that the Kv2.1 protein contributes to one or more of these channels. While three of these channels exhibit properties similar to delayed rectifierlike channels, none perfectly match the properties of cloned Kv2.1 channels expressed in *Xenopus* oocytes (Frech et al., 1989; VanDongen et al., 1990; Hartmann et al., 1991). It could be that the endogenous expression of Kv2.1 in PC12 cells yields channels with properties subtly different from those expressed in amphibian oocytes, due to different cellular biosynthetic or modulatory backgrounds. Alternatively, because K^+ channels probably exist as tetramers (MacKinnon, 1991), the differences could arise from heterotetrameric channels containing Kv2.1 polypeptide subunits in varying combinations or stoichiometries. Surprisingly, in one published report on the effects of NGF on K^+ channels, little or no change in the amplitude or macroscopic kinetics of whole PC12 cell K^+ current was observed upon NGF treatment (Garber et al., 1989). However, in this study, recordings were made from cells treated specifically to remove cellular processes in an effort to improve voltage-clamp analysis, either by growing the cells in suspension or by triturating the cells through a fine needle. As much of the Kv2.1 protein in NGF-differentiated PC12 cells is in the growth cone membranes, these treatments may have selectively removed many of the Kv2.1 channels whose expression is induced by NGF.

In an earlier study on the effects of NGF on PC12 ionic currents, O'Lague and colleagues (1985) specifically recorded from the growth cones of NGF-treated cells. Outside-out patches of growth cone membranes (O'Lague et al., 1985) contained low conductance K^+ channels similar to the delayed rectifier channels seen in patches excised from *Xenopus* oocytes microinjected with Kv2.1 cRNA (Hartmann et al., 1991). In addition, whole-cone recordings from isolated growth cones revealed macroscopic delayed rectifier currents (O'Lague et al., 1985) similar in kinetics and voltage dependence to Kv2.1 currents expressed in oocytes (Frech et al., 1989). It is interesting to speculate that these growth cone K^+ currents are due to the high level expression of Kv2.1 in this region, and that the lack of marked increases in K^+ currents in NGF-treated cells stripped of their neurites is due to the selective removal of the population of Kv2.1 expressed in growth cones. Future experiments utilizing overexpression and/or knockout of Kv2.1 expression in PC12 cells may lead to a better understanding of the role of this K^+ channel polypeptide in shaping the electrical properties of these cells.

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