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ENHANCED EXPRESSION OF Ca^{2+} CHANNELS BY NERVE GROWTH FACTOR AND THE v-*src* ONCOGENE IN RAT PHAEOCHROMOCYTOMA CELLS

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SUMMARY

1. Rat phaeochromocytoma (PC12) cells were used to investigate the expression of Ca^{2+} channel types during neuronal differentiation. Neuronal differentiation was induced by treatment with nerve growth factor (NGF) or by activation of a temperature-sensitive tyrosine kinase ($pp60^{v\text{-}src}$) in genetically modified PC12 (PC12/v-*src*) cells. PC12 cells differentiated morphologically in the presence of NGF. When grown at the permissive temperature of 37 °C which activates the kinase activity of $pp60^{v\text{-}src}$, PC12/v-*src* cells differentiated morphologically with the extension of neurites. In contrast, PC12/v-*src* cells grown at the non-permissive temperature of 40 °C continued to divide and were morphologically indistinguishable from control PC12 cells.

2. Whole-cell Ca^{2+} currents were measured in PC12 cells using Ba^{2+} as the charge carrier. Ba^{2+} currents measured at the peak of the current–voltage curve from a holding potential of −80 mV were $-0{\cdot}28 \pm 0{\cdot}04$ nA (mean ± S.E.M.) in control PC12 cells compared to $-1{\cdot}25 \pm 0{\cdot}16$ nA in NGF-differentiated cells. The current density increased from $9{\cdot}4 \pm 0{\cdot}7$ pA/pF in control PC12 cells to $22{\cdot}8 \pm 2{\cdot}4$ pA/pF in NGF-differentiated PC12 cells. Ba^{2+} currents were $-0{\cdot}24 \pm 0{\cdot}04$ nA in undifferentiated PC12/v-*src* cells grown at the non-permissive temperature of 40 °C compared to $-0{\cdot}95 \pm 0{\cdot}16$ nA in differentiated PC12/v-*src* cells grown at the permissive temperature of 37 °C. The current density increased from $4{\cdot}5 \pm 0{\cdot}5$ pA/pF in PC12/v-*src* cells grown at the non-permissive temperature of 40 °C to $13{\cdot}3 \pm 2{\cdot}4$ pA/pF in PC12/v-*src* cells grown at the permissive temperature of 37 °C.

3. The sensitivity of Ba^{2+} currents to ω-conotoxin GVIA (ω-CgTX) was determined for currents measured at the peak of the current–voltage curve (0 mV in 10 mM Ba^{2+}) from a holding potential of −80 mV. In NGF-differentiated PC12 cells, 10 μM ω-CgTx inhibited $68{\cdot}1 \pm 3{\cdot}2$% of the total Ba^{2+} current compared to $35{\cdot}9 \pm 4{\cdot}1$% in control cells. The density of the ω-CgTX-sensitive current increased from $3{\cdot}3 \pm 0{\cdot}4$ pA/pF in control cells to $15{\cdot}7 \pm 2{\cdot}0$ pA/pF in NGF-differentiated cells. In

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differentiated PC12/v-*src* cells grown at 37 °C, ω-CgTX inhibited 52·2 ± 4·2 % of total Ba^{2+} current compared to 41·1 ± 3·8 % in PC12/v-*src* cells grown at 40 °C. The density of the ω-CgTX-sensitive current increased from 1·9 ± 0·3 to 7·4 ± 2·0 pA/pF with v-*src*-mediated differentiation.

4. Ba^{2+} currents were separated into three components based upon their inhibition by Ca^{2+} channel antagonists. Ba^{2+} currents were measured at the peak of the current–voltage curve from a holding potential of −60 mV. Sequential additions of ω-CgTX and nifedipine separated the Ba^{2+} currents into three components: ω-CgTX sensitive, nifedipine sensitive and ω-CgTX and nifedipine resistant. The Ba^{2+} current in control PC12 cells was 27·2 ± 4·0 % ω-CgTX sensitive, 33·8 ± 1·1 % nifedipine sensitive and 39·0 ± 2·4 % resistant. In NGF-differentiated PC12 cells, the Ba^{2+} current was 55·8 ± 5·3 % ω-CgTX sensitive, 8·4 ± 2·5 % nifedipine sensitive and 35·8 ± 6·0 % resistant. Similarly, differentiated PC12/v-*src* cells grown at 37 °C had a Ba^{2+} current which was 50·3 ± 4·7 % ω-CgTX sensitive, 8·0 ± 4·6 % nifedipine sensitive and 41·7 ± 3·6 % resistant. Control PC12/v-*src* cells grown at 40 °C had a Ba^{2+} current which was 30·2 ± 4·6 % ω-CgTX sensitive, 46·1 ± 5·8 % nifedipine sensitive and 23·7 ± 4·8 % resistant.

5. Binding of ^{125}I-ω-CgTX increased 6-fold in PC12 cells treated with NGF for 6 days compared to control cells. Differentiated PC12/v-*src* cells grown at 37 °C showed a 4-fold increase in ^{125}I-ω-CgTX binding compared to control PC12/v-*src* cells grown at 40 °C. Nifedipine did not displace ^{125}I-ω-CgTX binding indicating independent binding sites.

6. Electrophysiological, pharmacological and radioligand binding studies indicate an enhanced expression of ω-CgTX-sensitive N-type Ca^{2+} channels in both NGF-treated and v-*src* differentiated PC12 cells. Additionally, a current component resistant to block by both ω-CgTX and nifedipine also showed an enhanced density in both NGF-treated and v-*src*-differentiated PC12 cells. The contribution of the nifedipine-sensitive L-type current to the total whole-cell current decreased in both NGF-treated and v-*src*-differentiated PC12 cells.

INTRODUCTION

Neurotrophic factors, such as nerve growth factor (NGF) and brain-derived neurotrophic factor, promote the survival of largely non-overlapping populations of neuronal cells (Thoenen, 1991). Investigations into the cellular mechanisms of growth factor-induced neuronal differentiation have employed PC12 cells, a rat phaeochromocytoma cell line, as a model system. PC12 cells extend neurites and differentiate into sympathetic neurone-like cells in the presence of NGF (Greene & Tischler, 1982). Differentiated PC12 cells become electrically excitable, that is, they acquire voltage-dependent Na^+ and Ca^{2+} channels and are, therefore, capable of a regenerative response (Dichter, Tischler & Greene, 1977; Rudy, Kirschenbaum, Rukenstein & Greene, 1987; Streit & Lux, 1987; Mandel, Cooperman, Maue, Goodman & Brehm, 1988). The acquisition of Ca^{2+} channels, in particular, endow neurones with the ability to control secretion of neurotransmitters (Hirning *et al.* 1988; Miller, 1990) and gene transcription (Morgan & Curran, 1986). However, neuronal Ca^{2+} channels exist as diverse types and just which types of Ca^{2+} channels a neurone expresses may determine its role in neuronal function.

The best studied neuronal growth factor is NGF. The high-affinity NGF receptor is composed of the *trk* proto-oncogene tyrosine kinase receptor and a 75 kDa low-affinity receptor (Hempstead, Martin-Zanca, Kaplan, Parada & Chao, 1991; Kaplan, Hempstead, Martin-Zanca, Chao & Parada, 1991). NGF has been shown to induce tyrosine phosphorylation and tyrosine kinase activity of the *trk* proto-oncogene product, and NGF responsiveness is restored with *trk* cDNA in NGF-non-responsive PC12 cells (Kaplan, Martin-Zanca & Parada, 1991; Loeb, Maragos, Martin-Zanca, Chao, Parada & Greene, 1991). This suggests that the tyrosine kinase activity of *trk* induced by NGF may play an essential role in the cellular events leading to neuronal differentiation.

Another tyrosine kinase, the product of the v-*src* oncogene, mimics certain characteristics of NGF-induced neuronal differentiation in PC12 cells (Alema, Casalbore, Agostini & Tato, 1985; Maher 1988; Rausch *et al.* 1989; Thomas *et al.* 1991). The following evidence suggests that the *src* tyrosine kinase may play a role in neuronal differentiation: (i) the existence of a neurone-specific, alternatively spliced c-*src* mRNA (Levy, Dorai, Wang & Brugge, 1987; Martinez, Mathey-Prevot, Bernards & Baltimore, 1987), (ii) the localization of this form of the *src* protein to specific neurones (Brugge, Cotton, Querral, Barrett, Nonner & Keane, 1985) and (iii) an increase in the *src* protein at the onset of neuronal differentiation in chick retinal cells (Sorge, Levy & Maness 1984).

Because little is known of the factors and mechanisms that control the expression of ion channels during development of the nervous system, we have begun to study the effects of tyrosine kinases on the expression of distinct Ca^{2+} channel types using PC12 cells as a model system. Ca^{2+} channels have been classified electrophysiologically and pharmacologically as L, N, T and P types (Nowycky, Fox & Tsien, 1985; Llinás, Sugimori, Lin & Cherksey, 1989). L-type Ca^{2+} channels are high voltage-activated channels which are sensitive to dihydropyridines. N-type Ca^{2+} channels are high voltage-activated channels which are insensitive to dihydropyridines but sensitive to the snail toxin, ω-conotoxin GVIA (ω-CgTX) (Plummer, Logothetis & Hess, 1989). T-type channels are low voltage-activated channels. P-type Ca^{2+} channels which are found in neurones of the cerebellum are insensitive to both dihydropyridines and ω-CgTX, but are inhibited by a polyamine fraction and a polypeptide, ω-Aga IVA, from *Agelenopsis aperta* venom (Llinás *et al.* 1989; Mintz, Venema, Swiderek, Lee, Bean & Adams, 1992).

PC12 cells differentiated with NGF express both L- and N-type Ca^{2+} channels but show a preferential increase in N-type channels with differentiation (Takahashi, Tsukui & Hatanaka, 1985; Plummer *et al.* 1989; Rausch, Lewis, Barker & Eiden, 1990; Usowicz, Porzig, Becker & Reuter, 1990). We now report that neuronal differentiation of PC12 cells by v-*src* results in a similar preferential increase in the density of ω-CgTX-sensitive N-type Ca^{2+} channels. In addition, the density of current resistant to block by N- and L-type channel blockers was enhanced in both NGF-treated and v-*src*-differentiated PC12 cells. Finally, both NGF- and v-*src*-differentiated PC12 cells showed a decrease in the proportion of current carried through L-type Ca^{2+} channels.

METHODS

Cell culture

PC12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 7% fetal bovine serum, 7% horse serum and 25 mM Hepes (pH 7·4). NGF was added to the media at a concentration of 50 ng/ml. Cells were fed every 3 days and grown in 7·5% CO_2 at 37 °C. Retroviral-mediated gene transfer was used to establish a PC12 cell line containing a temperature-sensitive v-*src* gene, ts NY68 (Kawai & Hanafusa, 1971; Rausch *et al.* 1990). The v-*src* expressing PC12 cell line (PC12/v-*src*) was carried at the non-permissive temperature of 40 °C during which the cells continue to divide. When switched to 37 °C to activate the kinase activity of the temperature-sensitive tyrosine kinase ($pp60^{v\text{-}src}$) the PC12/v-*src* cells stopped dividing and extended neurites. PC12 cells were plated onto poly-D-lysine-coated 35 mm dishes (Corning) and grown under four conditions: control PC12 cells, NGF-treated PC12 cells, PC12/v-*src* cells grown at the non-permissive temperature of 40 °C and PC12/v-*src* cells grown at the permissive temperature of 37 °C.

Patch-clamp recordings

Whole-cell recordings (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) were made on PC12 cells bathed in external medium containing (mM): 155 Tris, 10 Hepes, 5 $BaCl_2$, 5 barium acetate, 10 TEA acetate, 10 glucose, pH 7·4 with methanesulphonic acid (314 mosmol $(kg\ H_2O)^{-1}$). Patch pipettes (Corning 7052 glass, Garner Glass Co., Claremont, CA, USA) were fabricated on a model P-87 Flaming/Brown micropipette puller, coated with Sylgard® 184 (Dow Corning, Midland, MI, USA) and fire polished. Patch pipettes were filled with an intracellular solution (mM): 130 *N*-methyl-D-glucamine, 10 Hepes, 4 $MgCl_2$, 10 TEA acetate, 2 HCl, 5 BAPTA (1,2-bis(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid), 5 Tris ATP, 0·1 Na_2GTP, pH 7·2 with Tris base and methanesulphonic acid (300 mosmol $(kg\ H_2O)^{-1}$). Patch pipettes had resistances of 2–3 MΩ in the above solutions. The series resistance in the whole-cell mode was 3–6 MΩ. Membrane capacitance was determined from the time constant and area of the uncompensated capacitive transient. Fast transients in the cell-attached mode and capacitative transients in the whole-cell mode were cancelled. Series resistance compensation was > 80%. Recordings on all cell types were done at room temperature (22–25 °C). Cells with large, extensive neurites are difficult to properly clamp; therefore, only cells with neurites that were of sufficiently short electrotonic length to permit voltage control were studied. Adequate voltage control was determined by the symmetrical nature of the current–voltage relationship.

Whole-cell Ba^{2+} currents conducted through Ca^{2+} channels were recorded with an Axopatch 1-D (Axon Instruments, Foster City, CA, USA) patch-clamp amplifier, filtered at 2 kHz (−3 dB, 4-pole Bessel) and digitized at 200 μs per point. Voltage pulse protocols were generated and voltage and current traces digitized using a Macintosh IIcx computer (Apple Computer, Cupertino, CA, USA) equipped with a MacAdios II data acquisition board (GW Instruments Inc., Cambridge, MA, USA) using software written by Dr Stephen R. Ikeda (S3©, Medical College of Georgia, Augusta, GA, USA). Synthetic ω-CgTX (10 μM) was applied by superfusion from a large-bore pipette lowered into the bath to within 10 μm of the cell under study. The dihydropyridine antagonist, nifedipine (10 μM) was superfused by bath perfusion.

Current traces were analysed using Igor (WaveMetrics, Lake Oswego, OR, USA). Graphs and current traces were produced on a Macintosh IIci computer and LaserWriter IINT printer (Apple Computer) using Canvas (Deneba Software, Miami, FL, USA), DeltaGraph (DeltaPoint, Inc., Monterey, CA, USA), Igor (WaveMetrics) and software written by Dr Stephen R. Ikeda. Ba^{2+} current amplitudes were determined isochronally 10 ms after the onset of the test pulse. Current–voltage (*I–V*) relationships were corrected for linear leakage as determined from hyperpolarized test potentials. Data are presented as means ± S.E.M. where appropriate. Statistical differences are calculated using Student's *t* test.

Binding experiments with ^{125}I-ω-conotoxin GVIA

Membrane solubilization. Cells were displaced from culture dishes by incubation with trypsin–versene (0·1% w/v trypsin plus 0·02% w/v disodium EDTA) for 10 min at 37 °C in a 7·5% CO_2 incubator. Washed cells (twice in 10 ml of Ca^{2+}/Mg^{2+}-free phosphate-buffered saline, pH 7·4) were collected by centrifugation at 250 *g* for 5 min. The pellet was resuspended by gentle vortex

mixing and allowed to stand for 60 min at 4 °C in a double volume of extraction buffer (2% w/v CHAPS (3-((3-cholamidopropyl)-dimethylammonio)-1-propane sulphonate, v/v glycerol, 20 mM Tris, with the following protease inhibitors: 0·1 mM phenylmethylsulphonylfluoride, 1000 i.u./ml aprotinin, 0·01 mg/ml pepstatin A, with the pH adjusted to 7·5 with Hepes. Insoluble material was removed by centrifugation at 105000 *g* for 60 min in a Beckman SW-60 rotor, and the extract was assayed immediately. Protein determinations were by the method of Bradford (1976).

Binding assays

Extracts (20–100 μg protein), 10–200 fmol of ^{125}I-ω-CgTX and 10 μg of bovine serum albumin (BSA) were held in a final volume of 110 μl with 0·32 M sucrose, 5 mM Hepes/Tris, pH 7·4, 0·1 M NaCl, for 60 min at ~ 20 °C. For measurement of the non-saturable binding component, a 10- to 100-fold excess of the K_D (dissociation constant) of unlabelled ω-CgTX was preincubated at 4 °C for 30 min with a control aliquot of cell extract before adding ^{125}I-ω-CgTX. Bound radioactivity was recovered by filtration through Whatman GF/B filters presoaked (2 h at 4 °C) with 1% polyethyleneimine followed by washing 4 times with 4 ml of ice-cold 50 mM Tris-HCl at pH 7·4. Filters were counted and specific binding defined as the difference between total counts bound in the absence of excess unlabelled toxin and counts recovered from the non-saturable tubes containing excess unlabelled toxin.

Displacement experiments

^{125}I-ω-CgTX binding was determined in the presence of nifedipine (10^{-4} M), ω-conotoxin GVIA, conantokin G and conotoxin GIII in order to determine their interaction with the ω-CgTX GVIA binding site and the specificity of ω-CgTX GVIA. Toxins were used at 25-fold excess of the K_D, unless otherwise stated.

Materials

Synthetic ω-conotoxin GVIA was purchased from Peninsula Laboratories, Belmont, CA, USA. ^{125}I-ω-conotoxin GVIA was purchased from Amersham (specific activity 2200 Ci/mmol). Nifedipine, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulphonic acid (Hepes), Trizma base, protease (*S. aureus*, strain V8), glycerol, proteolytic inhibitors, Tris ATP and Na_2GTP were from Sigma (St Louis, MO, USA). CHAPS was from Boehringer Mannheim, Indianapolis, IN, USA. Nerve growth factor was isolated according to the procedure of Bocchini & Angeletti (1969).

RESULTS

Morphology

Control PC12 cells continued to divide and their somata remained small over 6 days in culture (Fig. 1*A*). PC12 cells treated with NGF (50 ng/ml) for 6 days differentiated morphologically into neurone-like cells. Cell division was inhibited, cell soma size increased and long, branching neurites were observed (Fig. 1*B*). PC12/v-*src* cells grown at the non-permissive temperature of 40 °C were morphologically indistinguishable from control PC12 cells (Fig. 1*C*). PC12/v-*src* cells grown at the permissive temperature of 37 °C for 6 days ceased to divide, increased in size and extended neurites (Fig. 1*D*).

Binding of ^{125}I-ω-conotoxin to PC12 lysates

Specific ^{125}I-ω-conotoxin GVIA binding to cell lysates increased in PC12 cells grown in the presence of NGF for 3-10 days (Fig. 2). The increase in ^{125}I-ω-CgTX binding was evident at 3 days of NGF treatment. ^{125}I-ω-CgTX binding increased in PC12/v-*src* cells grown at 37 °C from 6 to 10 days, lagging behind NGF-treated cells. In control PC12 cells, no increase in binding was seen until 10 days. In PC12/v-*src* cells grown at 40 °C there was no change in ^{125}I-ω-CgTX binding over 10 days. The

greatest difference in ^{125}I-ω-conotoxin binding between differentiated and non-differentiated PC12 cells was seen on day 6; therefore, all binding and electrophysiological experiments were done on days 5–6.

Figure 3 shows the saturation isotherm for the specific binding of ^{125}I-ω-conotoxin per mg of protein in PC12 cells after 6 days in culture. The K_D values (estimated from

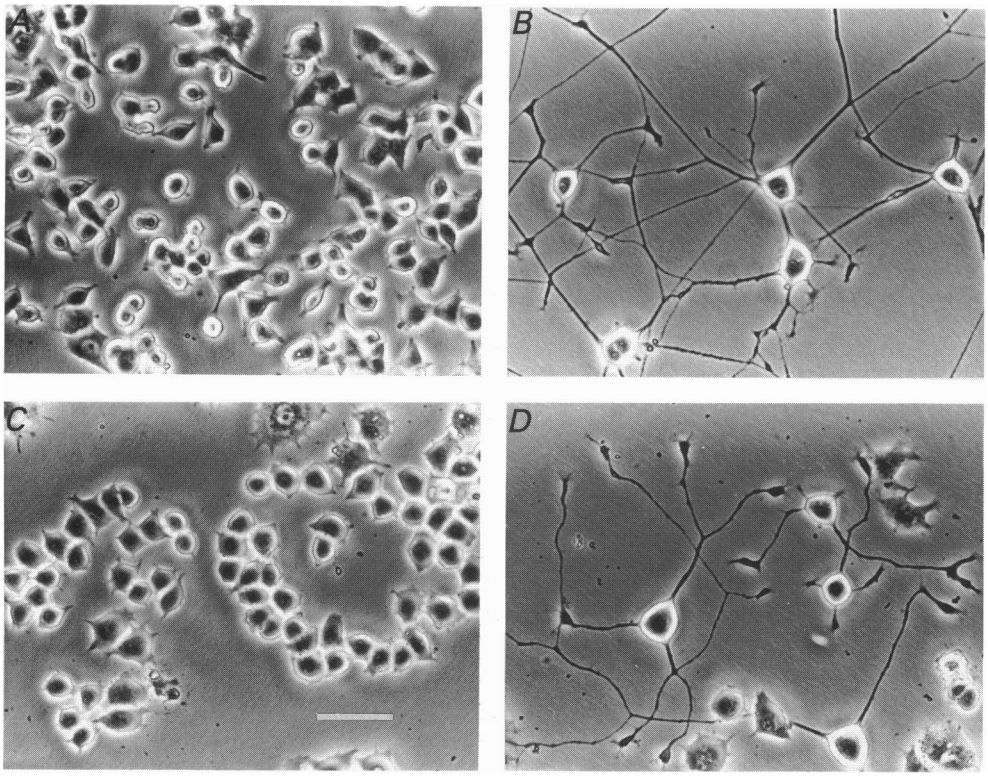

Fig. 1. Morphology of PC12 cell lines. *A*, control PC12 cells. *B*, PC12 cells treated with NGF. *C*, PC12/v-*src* cells grown at 40 °C. *D*, PC12/v-*src* cells grown at 37 °C. All cells were grown for 6 days. Note the increase in soma size and neurite outgrowth in NGF-treated PC12 cells and in PC12/v-*src* cells grown at the permissive temperature of 37 °C. Phase contrast optics. Calibration bar is approximately 50 μm.

the half-maximal saturation values) for control PC12 cells, NGF-treated PC12 cells, PC12/v-*src* cells grown at 40 °C and PC12/v-*src* cells grown at 37 °C were 1·7, 0·5, 1·4 and 0·7 nM, respectively, and the B_{max} (maximum binding capacity) values were 0·5, 1·4, 0·6 and 1·2 pmol/mg protein, respectively.

The specificity of the PC12 binding site for ^{125}I-ω-CgTX GVIA was analysed in the presence of the following compounds: conantokin G, a toxin reported to bind to the NMDA receptor (Olivera *et al.* 1990), conotoxin GIII, a μ-conotoxin that blocks skeletal muscle Na^+ channels (Olivera *et al.* 1990), and nifedipine, a dihydropyridine Ca^{2+} channel antagonist. The ^{125}I-ω-CgTX GVIA specific binding component was approximately 90% inhibited only by an excess of unlabelled ω-CgTX GVIA.

Whole-cell Ba^{2+} currents in differentiated vs. undifferentiated PC12 cells

Whole-cell Ba^{2+} currents were enhanced in PC12 cells after either NGF- or v-*src*-induced neurite outgrowth. Whole-cell Ba^{2+} currents were evoked from a holding potential of −80 mV to test potentials from −100 to +80 mV to generate a

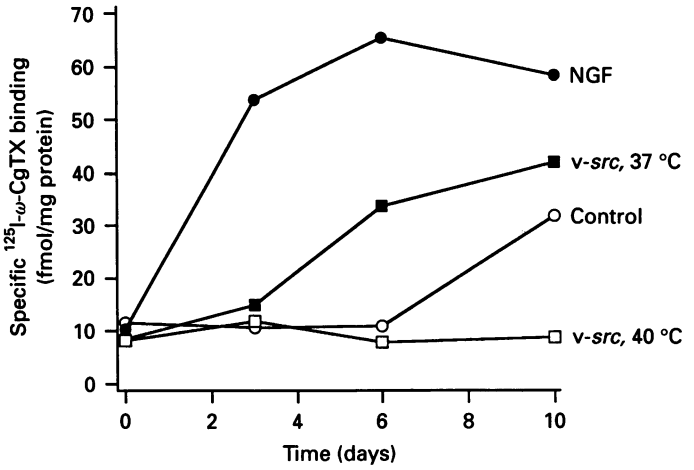

Fig. 2. Time course of specific ^{125}I-ω-CgTX binding to membrane extracts of undifferentiated and differentiated PC12 cells. PC12 cell extracts were assayed for specific ^{125}I-ω-CgTX binding (defined as the difference between total counts in the absence and presence of excess unlabelled toxin). Membrane extracts contained 25 μg protein per tube and were incubated with 20 fmol ^{125}I-ω-CgTX for 60 min at approximately 20 °C. PC12 cells differentiated by treatment with NGF (NGF; ●), showed an increase in specific ^{125}I-ω-CgTX binding by day 3 which remained elevated over 10 days. PC12/v-*src* cells grown at the permissive temperature of 37 °C (v-*src*, 37 °C; ■) showed a more gradual and smaller maximal increase in ^{125}I-ω-CgTX binding compared to the NGF-treated PC12 cells. Control, undifferentiated PC12 cells (Control; ○) showed no change in ^{125}I-ω-CgTX binding after 6 days in culture, but showed an increase after 10 days in culture. PC12/v-*src* cells grown at the non-permissive temperature of 40 °C showed no change in ^{125}I-ω-CgTX binding over 10 days in culture (v-*src*, 40 °C; □). Each point is the average of two experiments with coefficients of variation ranging from 5 to 17%.

current–voltage relationship in four types of PC12 cells: (i) control PC12 cells, (ii) NGF-treated PC12 cells, (iii) PC12/v-*src* cells grown at the non-permissive temperature of 40 °C and (iv) PC12/v-*src* cells grown at the permissive temperature of 37 °C. Figure 4 illustrates the increase in the Ba^{2+} current amplitude in PC12 cells after either NGF- or v-*src*-induced neurite outgrowth. The Ba^{2+} current amplitude at the peak of the current–voltage curve was −0·35 nA for a control PC12 cell (Fig. 4*A*), −2·22 nA for a PC12 cell treated with NGF (Fig. 4*B*), −0·48 nA for a PC12/v-*src* cell grown at 40 °C (Fig. 4*C*) and −2·14 nA for a PC12/v-*src* cell grown at 37 °C (Fig. 4*D*).

The onset of Ba^{2+} currents occurred at test potentials greater than −40 mV in both undifferentiated and differentiated PC12 cell types (Fig. 4). Therefore, PC12 cells did not express low-threshold, T-type Ca^{2+} channels, but only high voltage-activated Ca^{2+} channels, in agreement with earlier studies (Streit & Lux, 1987; Plummer *et al.* 1989; Rausch *et al.* 1990; Usowicz *et al.* 1990).

Pharmacology of Ba^{2+} currents in differentiated vs. undifferentiated PC12 cells

Augmentation of the Ba^{2+} current amplitude with differentiation induced by NGF has been shown to be due to an increase in the number of N-type Ca^{2+} channels (Plummer *et al.* 1989; Rausch *et al.* 1990; Usowicz *et al.* 1990). We tested whether N-

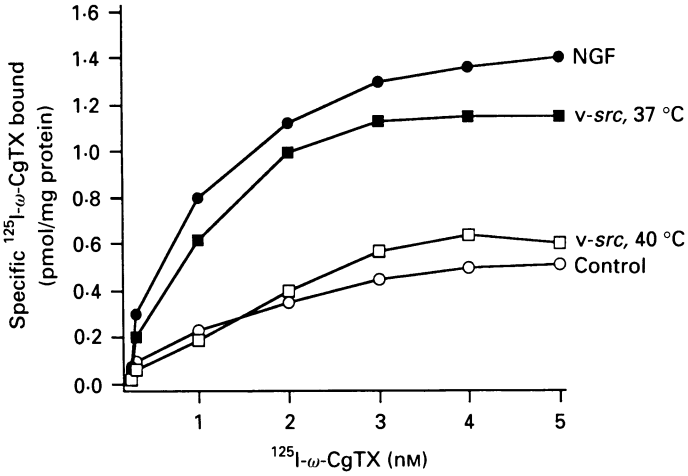

Fig. 3. Saturation isotherm for ^{125}I-ω-CgTX binding to PC12 cells. Specific ^{125}I-ω-CgTX binding was assayed for detergent extracts of PC12 cells after 6 days in culture. The K_D was estimated from the half-maximal saturation and was 0·5 nM for NGF-treated PC12 cells (NGF; ●) and 0·47 nM for PC12/v-*src* cells grown at the permissive temperature (v-*src*, 37 °C; ■). The K_D for control PC12 cells (Control; ○) was 1·4 nM and for control PC12/v-*src* cells grown at the non-permissive temperature of 40 °C (v-*src*, 40 °C; □) it was 1·7 nM. The NGF-treated and the PC12/v-*src* cells grown at 37 °C have identical K_D values which are lower than the K_D values for the undifferentiated control PC12 cells and the PC12/v-*src* cells grown at 40 °C. This may suggest that a high affinity ^{125}I-ω-CgTX binding site has been up-regulated during differentiation induced by both NGF and $pp60^{v\text{-}src}$.

type Ca^{2+} channels show enhanced expression during NGF- or v-*src*-induced differentiation. We also compared the functional expression of L-type Ca^{2+} channels during neuronal differentiation. Changes in the expression of a particular type of Ca^{2+} channel can be assessed by pharmacological means. We used ω-CgTX, which selectively blocks N-type channels (Plummer *et al.* 1989), and the dihydropyridine antagonist nifedipine, which selectively blocks L-type channels (Bean, 1984; Sanguinetti & Kass, 1984) to assess the contribution of these channel types to the increased amplitude of the whole-cell Ba^{2+} currents during two types of neuronal differentiation. If neuronal differentiation enhances the expression of N-type channels, we would predict that a greater proportion of Ba^{2+} current in the differentiated PC12 cells would be sensitive to block by ω-CgTX.

ω-CgTX

Current–voltage curves were elicited from PC12 cells voltage clamped to a holding potential of −80 mV. Figure 4 shows the effect of ω-CgTX (ω-CgTX GVIA, 10 μM) on the current–voltage curves of representative cells. The peak Ba^{2+} current amplitude

was reduced from −0·35 to −0·26 nA in the presence of ω-CgTX in a control PC12 cell (Fig. 4*A*). In a PC12 cell treated with NGF (Fig. 4*B*), the peak Ba^{2+} current amplitude was reduced from −2·22 to −0·97 nA in the presence of ω-CgTX. ω-CgTX decreased the Ba^{2+} current amplitude from −0·48 to −0·25 nA in an undifferentiated

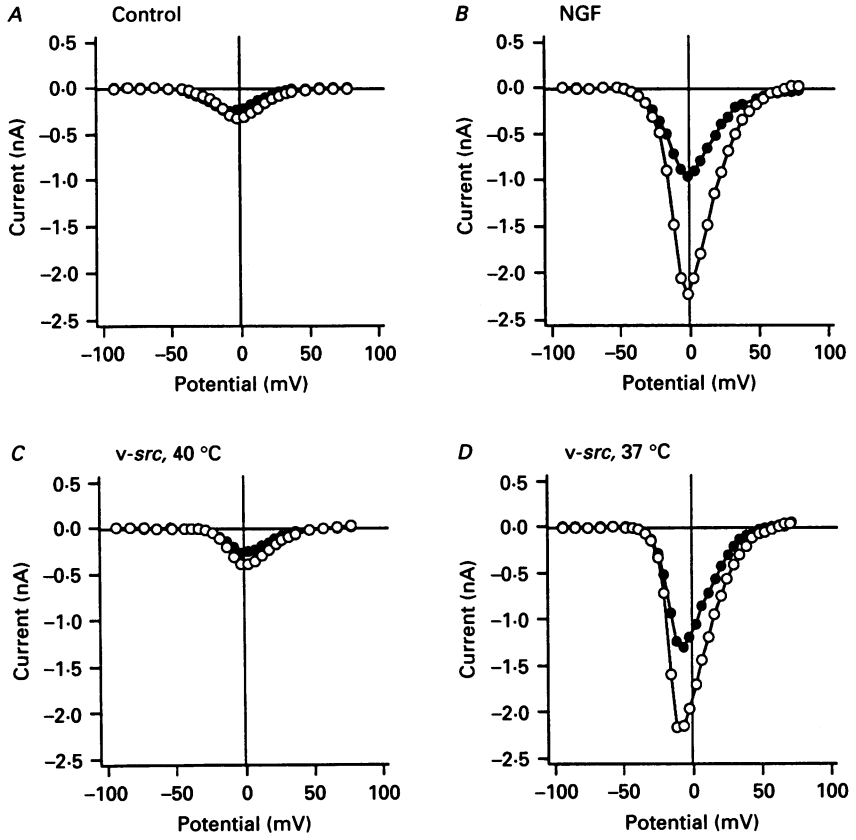

Fig. 4. Differentiation of PC12 cells by either NGF or $pp60^{v\text{-}src}$ increases the component of whole-cell Ba^{2+} current that can be blocked by ω-CgTX. Currents were elicited by depolarizing voltage steps to various test potentials from a holding potential of −80 mV. Current–voltage relationships of the amplitude of the Ba^{2+} current measured isochonally 10 ms after the voltage step to the indicated test potential are shown both in the absence (○) and presence (●) of 10 μM ω-CgTX for four types of PC12 cells. *A*, a control PC12 cell 5 days in culture. *B*, a PC12 cell treated for 6 days with NGF. *C*, an undifferentiated PC12/v-*src* cell grown at the non-permissive temperature of 40 °C for 5 days. *D*, a differentiated PC12/v-*src* cell grown at the permissive temperature of 37 °C for 6 days. Note the large increase in amplitude of the Ba^{2+} currents in both differentiated cell types (*B* and *D*).

PC12/v-*src* cell grown at 40 °C (Fig. 4*C*). In a differentiated PC12/v-*src* cell grown at 37 °C (Fig. 4*D*), the peak Ba^{2+} current amplitude was reduced from −2·14 to −1·30 nA in the presence of ω-CgTX. Figure 5 shows the current traces at the peak of the current–voltage curves in the absence and presence of ω-CgTX for the cells shown in Fig. 4.

The mean amplitude and density for the total peak current and the ω-CgTX-

sensitive current in all four types of PC12 cells are shown in Fig. 6. The average peak Ba^{2+} current in control PC12 cells was $-0{\cdot}28 \pm 0{\cdot}04$ nA ($n = 9$), the mean whole-cell capacitance, C_m (a measure of cell surface area) was $29{\cdot}6 \pm 3{\cdot}5$ pF and the current density was $9{\cdot}4 \pm 0{\cdot}7$ pA/pF. NGF-induced differentiation of PC12 cells was

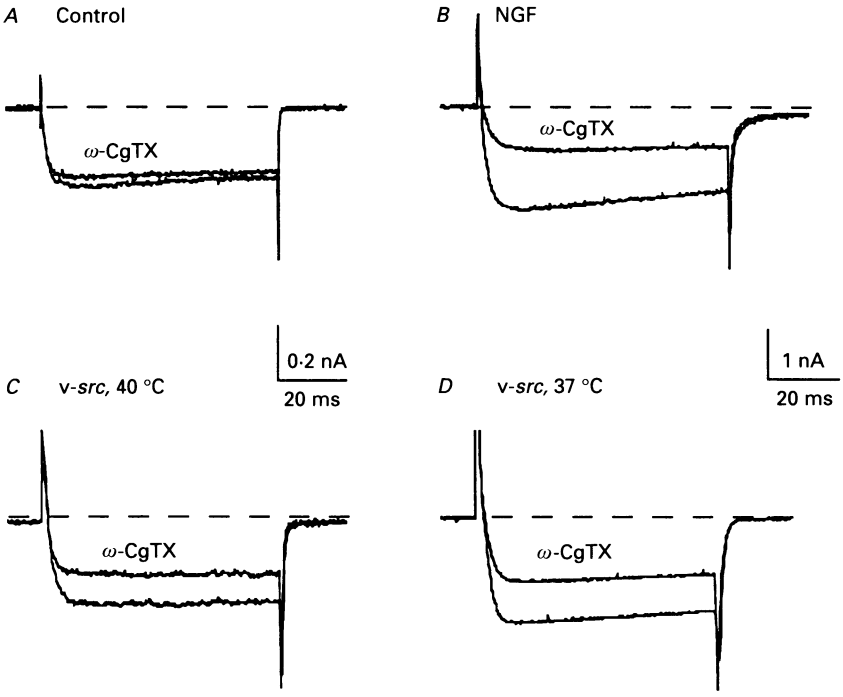

Fig. 5. ω-CgTX blocks a greater component of the whole-cell Ba^{2+} current in PC12 cells differentiated with NGF or $pp60^{v\text{-}src}$. Currents were elicited by a depolarizing voltage step to the peak of the current–voltage relationship from a holding potential of −80 mV. Currents are from the same cells shown in Fig. 4. Superimposed traces show the Ba^{2+} currents in the absence and presence of 10 μM ω-CgTX (trace labelled ω-CgTX). *A*, control PC12 cell; *B*, NGF-treated PC12 cell; *C*, PC12/v-*src* cell grown at the non-permissive temperature of 40 °C; *D*, PC12/v-*src* cell grown at the permissive temperature of 37 °C. Current calibration bar is 0·2 nA for *A* and *C* and 1 nA for *B* and *D*.

characterized by a significant increase in the amplitude and density of the Ba^{2+} current. In NGF-differentiated cells the average peak Ba^{2+} current increased to $-1{\cdot}25 \pm 0{\cdot}16$ nA ($n = 9$), the C_m was $56{\cdot}9 \pm 5{\cdot}2$ pF and the current density was $22{\cdot}8 \pm 2{\cdot}4$ pA/pF. Undifferentiated PC12/v-*src* cells grown at 40 °C had an average peak Ba^{2+} current of $-0{\cdot}24 \pm 0{\cdot}04$ nA ($n = 10$), a C_m of $53{\cdot}4 \pm 6{\cdot}1$ pF and a current density of $4{\cdot}5 \pm 0{\cdot}5$ pA/pF. Differentiated PC12/v-*src* grown at 37 °C showed a significant increase in the amplitude and density of the Ba^{2+} current. In differentiated PC12/v-*src* cells grown at 37 °C the average peak Ba^{2+} current was $-0{\cdot}95 \pm 0{\cdot}16$ nA ($n = 10$), the C_m was $77{\cdot}6 \pm 10{\cdot}1$ pF and the current density was $13{\cdot}3 \pm 2{\cdot}4$ pA/pF. Thus, a significant increase in both the current amplitude and the current density was seen during neuronal differentiation induced either by NGF treatment or by $pp60^{v\text{-}src}$.

ω-CgTX and nifedipine

Whole-cell Ca^{2+} currents in PC12 cells are likely to be composed of various types of Ca^{2+} channels. We used ω-CgTX and nifedipine, selective antagonists of N- and L-type Ca^{2+} channels, respectively, to determine the contribution of these two channel

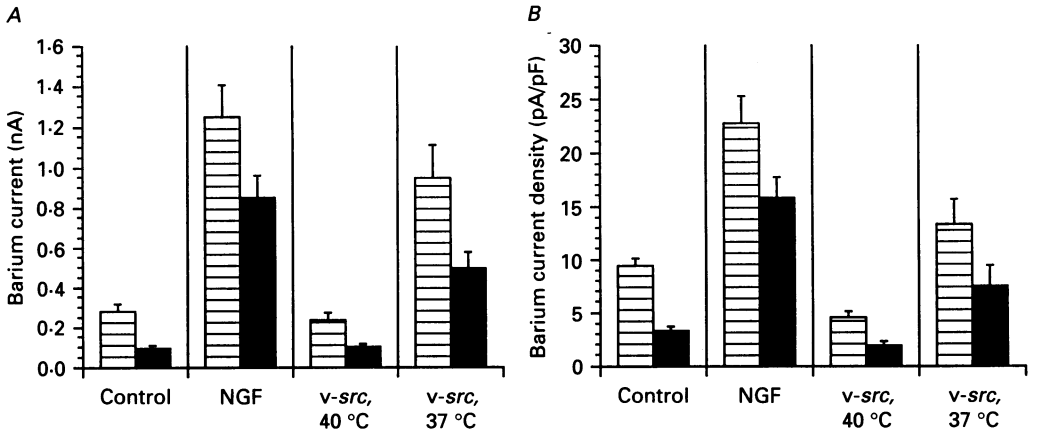

Fig. 6. Differentiation enhanced the amplitude and density of the total whole-cell Ba^{2+} current (bars with horizontal lines) and the ω-CgTX-sensitive current component (filled bars) in PC12 cells. Currents were elicited from a holding potential of −80 mV to a test potential at the peak of the current–voltage curve. *A*, NGF-differentiated PC12 cells (NGF) showed a significant increase in the amplitude of both the total whole-cell Ba^{2+} current ($P = 0{\cdot}0001$, $n = 9$) and in the ω-CgTX-sensitive current amplitude ($P = 0{\cdot}0001$) when compared to control PC12 cells (Control). PC12 differentiated by expression of v-*src* (v-*src*, 37 °C) also showed a significant increase in the amplitude of both the total whole-cell Ba^{2+} current ($P = 0{\cdot}0005$, $n = 10$) and in the ω-CgTX-sensitive current amplitude ($P = 0{\cdot}0002$) when compared to control v-*src* cells (v-*src*, 40 °C). *B*, the density of both the total whole-cell Ba^{2+} current and the ω-CgTX-sensitive current was significantly increased in PC12 cells differentiated with NGF (NGF, $P = 0{\cdot}0001$ for both, $n = 9$) when compared to control (Control). There was a significant increase in the density of both the total whole-cell Ba^{2+} current ($P = 0{\cdot}002$, $n = 10$) and the ω-CgTX-sensitive current ($P = 0{\cdot}01$) in PC12 cells differentiated by v-*src* expression (v-*src*, 37 °C) when compared to control v-*src* cells (v-*src*, 40 °C).

types to the whole-cell Ca^{2+} current in differentiated and undifferentiated PC12 cells. L-type Ca^{2+} channels were shown to be blocked by nifedipine in a voltage-dependent manner (Bean, 1984; Sanguinetti & Kass, 1984). Therefore, to facilitate the block by nifedipine, PC12 cells were voltage clamped to a holding potential of −60 mV. N-type Ca^{2+} channels were shown to be sensitive to holding potential but at a holding potential of −60 mV were only partially inactivated (Plummer *et al.* 1989). Thus, the contribution of N-type Ca^{2+} channels to the whole-cell current can be analysed by its sensitivity to ω-CgTX from a holding potential of −60 mV. By assaying the sequential effects of ω-CgTX and nifedipine on the whole-cell Ba^{2+} currents, three components could be defined, an ω-CgTX-sensitive component, a nifedipine-sensitive component and an ω-CgTX- and nifedipine-resistant component.

Voltage steps to the peak of the current–voltage relationship were elicited every 10 s until the Ba^{2+} current stabilized. ω-CgTX (10 μM), superfused from a pipette

lowered to within 10 μm of the cell, rapidly inhibited the Ba^{2+} current. Then, in the presence of ω-CgTX, nifedipine (10 μM) was superfused by bath perfusion. Figure 7 shows the sequential effect of ω-CgTX and nifedipine on the amplitude of the Ba^{2+} current for four PC12 cell types. In one control PC12 cell grown for 5 days (Fig. 7*A*)

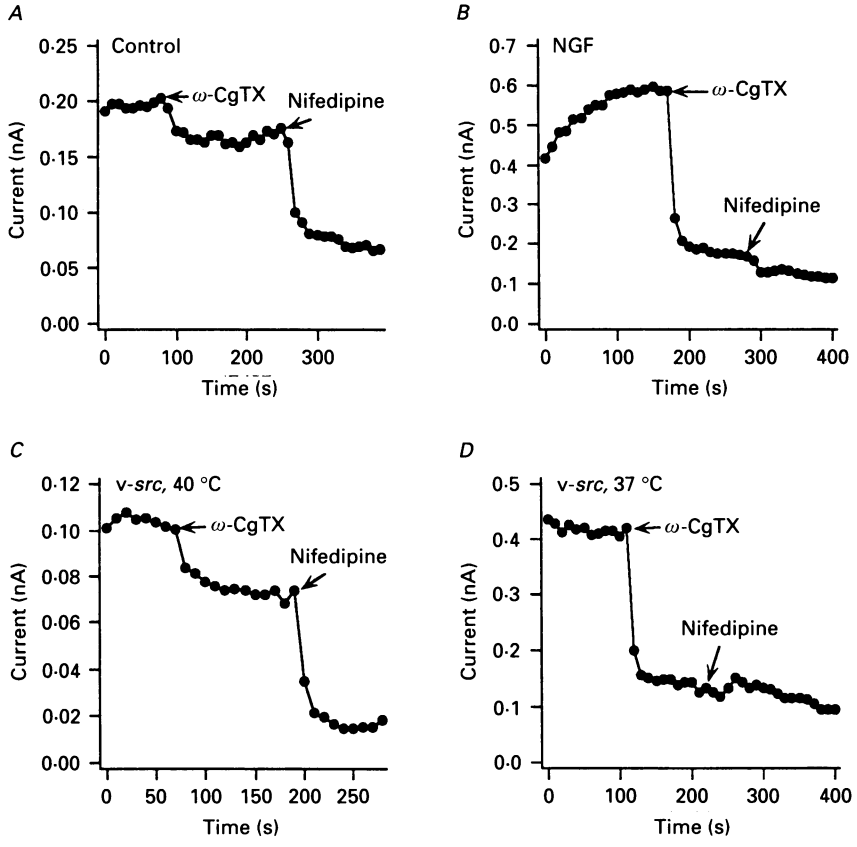

Fig. 7. The ω-CgTX-sensitive component of the whole-cell Ba^{2+} current is enhanced and nifedipine-sensitive current component is decreased in PC12 cells differentiated by NGF or $pp60^{v\text{-}src}$. Currents were elicited by a voltage step to 0 mV from a holding potential of −60 mV every 10 s. The effects of ω-CgTX (10 μM) applied by superfusion from a large bore pipette and nifedipine (10 μM) applied by bath superfusion were tested sequentially on the whole-cell Ba^{2+} currents in four PC12 cell types. *A*, a control PC12 cell 5 days in culture had a small component of whole-cell current that was sensitive to ω-CgTX, a large component that was inhibited by nifedipine and a component that was insensitive to both ω-CgTX and nifedipine. *B*, a PC12 cell treated with NGF for 5 days had a large component of whole-cell current that was sensitive to ω-CgTX, a component that was slightly inhibited by nifedipine and a component that was insensitive to both ω-CgTX and nifedipine. *C*, a PC12/v-*src* cell grown at the non-permissive temperature of 40 °C for 5 days had a small component of whole-cell current that was sensitive to ω-CgTX, a large component that was inhibited by nifedipine and a small component that was insensitive to both ω-CgTX and nifedipine. *D*, a PC12/v-*src* cell grown at the permissive temperature of 37 °C for 6 days had a large component of whole-cell current that was sensitive to ω-CgTX, a component that was insensitive to nifedipine and a component that was insensitive to both ω-CgTX and nifedipine.

the Ba^{2+} current was 16% ω-CgTX sensitive, 44% nifedipine sensitive and 40% resistant (i.e. not blocked by either ω-CgTX or nifedipine). In a similar PC12 cell treated with NGF for 5 days (Fig. 7*B*) the Ba^{2+} current was 58% ω-CgTX sensitive, 10% nifedipine sensitive and 32% resistant. A PC12/v-*src* grown at 40 °C for 5 days

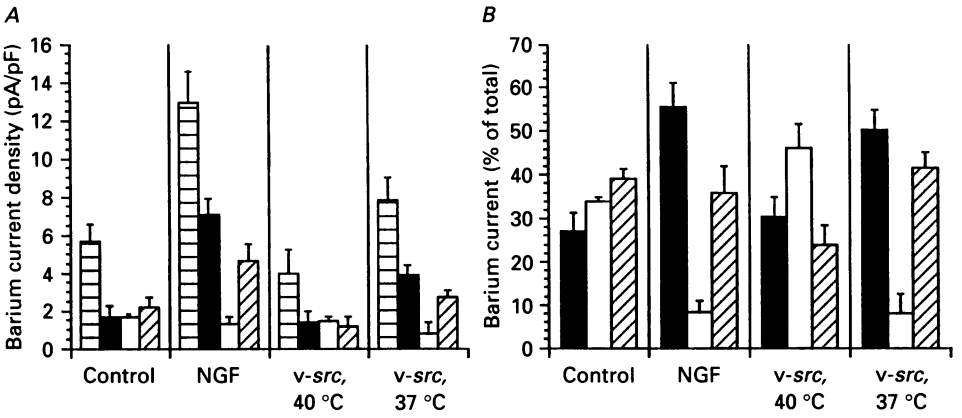

Fig. 8. Barium current components. Total whole-cell Ba^{2+} currents (bars with horizontal lines) elicited from a holding potential of −60 mV to a test potential at the peak of the current–voltage curve could be divided into an ω-CgTX-sensitive component (filled bars), a nifedipine-sensitive component (open bars), and a component resistant to both ω-CgTX and nifedipine (hatched bars) in four types of PC12 cells: control PC12 cells (Control, $n = 6$), NGF-treated PC12 cells (NGF, $n = 6$), control v-*src* PC12 cells grown at 40 °C (v-*src*, 40 °C, $n = 7$) and differentiated v-*src* PC12 cells grown at 37 °C (v-*src*, 37 °C, $n = 5$). *A*, the relative density of the components of the whole-cell Ba^{2+} currents. Differentiation of PC12 cells with NGF resulted in a significant increase in the density of total current ($P = 0{\cdot}003$), a significant increase in the density of the CgTX-sensitive component ($P = 0{\cdot}0003$), no change in the density of the nifedipine-sensitive component and a significant increase in the density of the component resistant to both ω-CgTX and nifedipine ($P = 0{\cdot}04$) when compared with control PC12 cells. Differentiated PC12/v-*src* cells (v-*src*, 37 °C) also showed a significant increase in the density of total current ($P = 0{\cdot}05$), a significant increase in the density of the CgTX-sensitive component ($P = 0{\cdot}01$), no change in the density of the nifedipine-sensitive component and a significant increase in the density of the component resistant to both ω-CgTX and nifedipine ($P = 0{\cdot}05$) when compared with control PC12/v-*src* cells (v-*src*, 40 °C). *B*, the relative percentage contribution of the components to the total whole-cell Ba^{2+} currents. PC12 cells treated with NGF showed a significant increase in the ω-CgTX-sensitive component ($P = 0{\cdot}001$) and a decrease in the nifedipine-sensitive component ($P = 0{\cdot}0007$) when compared with control PC12 cells. Likewise, differentiated PC12/v-*src* cells (v-*src*, 37 °C) showed a significant increase in the ω-CgTX-sensitive component ($P = 0{\cdot}01$) and a significant decrease in the nifedipine-sensitive component ($P = 0{\cdot}0007$) when compared to control PC12/v-*src* cells (v-*src*, 40 °C). Control PC12/v-*src* cells (v-*src*, 40 °C) showed a significant decrease in the Ba^{2+} current component resistant to both ω-CgTX and nifedipine ($P = 0{\cdot}02$) when compared to PC12/v-*src* cells grown at 37 °C.

(Fig. 7*C*) had a Ba^{2+} current that was 26% ω-CgTX sensitive, 60% nifedipine sensitive and 14% resistant. In one differentiated PC12/v-*src* cell grown at 37 °C for 6 days (Fig. 7*D*) the Ba^{2+} current was 65% ω-CgTX sensitive, 4% nifedipine sensitive and 31% resistant.

The total current density and the relative contribution of the components of the

Ba^{2+} current sensitive and resistant to ω-CgTX and nifedipine are shown in Figure 8*A*. The total whole-cell current density significantly increased in both NGF-treated ($P = 0{\cdot}003$) and v-*src*-differentiated PC12 cells ($P = 0{\cdot}05$) compared to control PC12 cells and control v-*src* 40 °C cells, respectively. The ω-CgTX-sensitive current density was significantly greater in NGF-differentiated cells ($P = 0{\cdot}0003$) compared to control PC12 cells and significantly greater in PC12/v-*src* cells grown at 37 °C ($P = 0{\cdot}01$) compared to PC12/v-*src* cells grown at 40 °C. The nifedipine-sensitive current density was not significantly different with differentiation in both PC12/NGF cells and PC12/v-*src* cells grown at 37 °C compared to their controls. The density of the Ba^{2+} current resistant to both ω-CgTX and nifedipine was significantly greater in NGF-treated PC12 cells ($P = 0{\cdot}04$) and in PC12/v-*src* cells grown at 37 °C ($P = 0{\cdot}05$) when compared to their controls. Figure 8*B* shows the Ba^{2+} current components expressed as a percentage of the total whole-cell Ba^{2+} current amplitude. The ω-CgTX-sensitive component showed a significantly enhanced contribution to the total current in NGF-differentiated PC12 cells ($P = 0{\cdot}001$) and in v-*src* 37 °C differentiated PC12 cells ($P = 0{\cdot}01$) when compared to their controls. The contribution of the nifedipine-sensitive component was significantly decreased in NGF-differentiated PC12 cells ($P = 0{\cdot}0007$) and in v-*src* differentiated PC12 cells ($P = 0{\cdot}0007$) when compared to their controls. In control v-*src* cells grown at 40 °C, the component resistant to block by both ω-CgTX and nifedipine was significantly decreased ($P = 0{\cdot}02$) when compared to differentiated v-*src* cells grown at 37 °C.

DISCUSSION

We have presented evidence from which the following conclusions can be drawn. (i) PC12 cells containing the temperature-sensitive NY68 v-*src* gene extend neurites in a temperature-sensitive manner under the control of the v-*src* tyrosine kinase which mimics the differentiation of PC12 cells into sympathetic-like neurones during NGF treatment. (ii) Both v-*src* tyrosine kinase expression and NGF treatment enhance the expression of ω-CgTX-sensitive N-type Ca^{2+} channels as evidenced by an increase in density of current through Ca^{2+} channels sensitive to ω-CgTX and by an increase in the specific binding of ^{125}I-ω-CgTX. (iii) Both v-*src* tyrosine kinase expression and NGF treatment enhanced the density of current through Ca^{2+} channels resistant to block by both ω-CgTX and nifedipine. (iv) The levels of expression of different Ca^{2+} channel types in PC12 cells induced by either v-*src* tyrosine kinase expression or NGF treatment mimic those found in mature sympathetic ganglion neurones. Our data suggest that transcription of specific Ca^{2+} channel types may be under the control of cellular signalling pathways activated by tyrosine kinases.

Both v-*src* expression and NGF treatment of PC12 cells have been shown to activate the transcription of many common genes suggesting a convergence of at least some of the signalling pathways involved in neuronal differentiation (Thomas *et al.* 1991). We speculate that some of these pathways may be involved in the enhanced expression of N-type Ca^{2+} channels in NGF-treated PC12 cells and in neurones of sympathetic and sensory ganglia whose survival is dependent upon NGF (Levi-Montalcini, 1987). Neurones of frog and rat sympathetic ganglia express Ca^{2+} channels primarily of the ω-CgTX-sensitive N-type (approximately 70% of the

whole-cell current) with a smaller component of current resistant to block by both ω-CgTX and dihydropyridines and an even smaller contribution from dihydropyridine-sensitive L-type Ca^{2+} channels (Hirning *et al.* 1988; Plummer *et al.* 1989; Jones & Jacobs, 1990; Ikeda, 1991). We have found that 68% of the whole-cell Ca^{2+} current in PC12 cells differentiated with NGF was ω-CgTX sensitive and that 52% of the whole-cell Ca^{2+} current in PC12 cells expressing a temperature-sensitive v-*src* protein was ω-CgTX sensitive. The remainder of the whole-cell current in NGF-treated and v-*src*-expressing PC12 cells was made up of a component resistant to both ω-CgTX and the dihydropyridine antagonist nifedipine (approximately 40%) and a component sensitive to nifedipine (8%). Thus, PC12 cells differentiated under our conditions of NGF treatment or v-*src* expression express a similar proportion of distinct Ca^{2+} channel types as those reported in mature sympathetic neurones.

The processes of neuronal differentiation induced a shift in the density of Ca^{2+} channel types. NGF treatment increased specific ^{125}I-ω-CgTX binding 6-fold and increased the density of ω-CgTX-sensitive N-type Ca^{2+} channels 5-fold in agreement with Usowicz *et al.* (1990). Differentiation of PC12 cells by $pp60^{v\text{-}src}$ resulted in a 4-fold increase in specific ^{125}I-ω-CgTX binding and a 3·4-fold increase in the density of N-type Ca^{2+} channels. The density of Ca^{2+} current resistant to both ω-CgTX and nifedipine increased in NGF-treated PC12 cells and in PC12/v-*src* cells grown at 37 °C when compared to control PC12 cells and PC12/v-*src* cells grown at 40 °C, respectively. It remains to be determined which Ca^{2+} channel types contribute to this resistant current component. The density of nifedipine-sensitive L-type Ca^{2+} channels showed little change in current density with differentiation.

The expression of neuronal N-type Ca^{2+} channels may be critical to neuronal function. N-type Ca^{2+} channels have been shown to play a role in secretion of neurotransmitters from certain neurones. Secretion of noradrenaline from PC12 cells was dihydropyridine sensitive in undifferentiated cells but changed to being primarily dihydropyridine insensitive in NGF-differentiated cells (Takahashi *et al.* 1985; Kongsamut & Miller, 1986). Secretion of noradrenaline from sympathetic neurones was shown to be insensitive to dihydropyridine blockers, but sensitive to block by ω-CgTX (Hirning *et al.* 1988). Ca^{2+} channel recordings from secretory chick ciliary ganglion presynaptic nerve terminals have shown that these channels are dihydropyridine insensitive and ω-CgTX sensitive (Stanley & Goping, 1991). Thus, the acquisition of N-type Ca^{2+} channels upon growth factor stimulation may endow specific neurones with the ability to control neurotransmitter secretion.

Activation of both the NGF receptor tyrosine kinase and the v-*src* tyrosine kinase in PC12 cells resulted in morphological differentiation and the acquisition of N-type Ca^{2+} channels. What are the similarities and differences in the cell signalling pathways activated by each of these tyrosine kinases? K252a, a protein kinase C inhibitor closely related to staurosporine, inhibited NGF-induced differentiation and c-*fos* transcription (Hashimoto, 1988; Koizumi, Contreras, Matsuda, Hama, Lazarovici & Guroff, 1988; Lazarovici *et al.* 1989), but did not inhibit v-*src*-induced differentiation in PC12 cells (Rausch *et al.* 1989). Staurosporine blocked both differentiation and enhancement of ω-CgTX binding sites in PC12 cells treated with NGF (Usowicz *et al.* 1990). K252a blocked NGF-induced tyrosine phosphorylation, but not EGF (epidermal growth factor)-induced tyrosine phosphorylation (Maher, 1989; Qui & Green, 1991) prompting Qui & Green (1991) to suggest that K252a may

be an inhibitor of NGF-stimulated protein tyrosine kinase and not a specific inhibitor of protein kinase C. The v-*src* protein may act in a different manner or at a convergent site in the NGF pathway. Both NGF and v-*src* show overlapping and specific tyrosine phosphorylation of several proteins including PLC-γ1 (Thomas *et al.* 1991). Tyrosine phosphorylation of PLC-γl was inhibited by K252a in NGF-treated, but not in v-*src*-expressing PC12 cells (Thomas *et al.* 1991). If the NGF receptor tyrosine kinase could activate *src*, this would indicate a single signalling pathway. Some evidence for this idea is that another receptor tyrosine kinase, platelet-derived growth factor, has been shown to activate c-*src* (Ralston & Bishop, 1985; Gould & Hunter, 1988; Kypta, Goldberg, Ulug & Courtneidge, 1990). Alternatively, v-*src* could mimic the action of *trk* and thus induce phosphorylation of certain substrates that feed into the differentiation pathway. This could account for the differences seen between NGF and v-*src*-induced differentiation (Rausch *et al.* 1989; Thomas *et al.* 1991).

We have shown that neuronal differentiation of PC12 cells induced by the activation of the NGF receptor tyrosine kinase or the v-*src* tyrosine kinase is accompanied by an enhanced expression of ω-CgTX-sensitive N-type Ca^{2+} channels and Ca^{2+} channels resistant to blockers of N- and L-type Ca^{2+} channels. There was also a decrease in the contribution of nifedipine-sensitive L-type Ca^{2+} channels to the total whole-cell current with differentiation by NGF or v-*src* tyrosine kinase.

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REFERENCES

ALEMA, S., CASALBORE, P., AGOSTINI, E. & TATO, F. (1985). Differentiation of PC12 phaeochromocytoma cells induced by v-*src* oncogene. *Nature* **316**, 557–559.

BEAN, B. P. (1984). Nitrendipine block of cardiac calcium channels: high affinity binding to the inactivated state. *Proceedings of the National Academy of Sciences of the USA* **81**, 6388–6392.

BOCCHINI, V. & ANGELETII, P. U. (1969). The nerve growth factor: purification as a 30,000 molecular weight protein. *Proceedings of the National Academy of Sciences of the USA* **64**, 787–794.

BRADFORD, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Analytical Biochemistry* **72**, 248–254.

BRUGGE, J. S., COTTON, P. C., QUERRAL, A. E., BARRETT, J. N., NONNER, D. & KEANE, R. W. (1985). Neurones express high levels of a structurally modified activated form of $pp60^{c\text{-}src}$. *Nature* **316**, 554–558.

DICHTER, M. A., TISCHLER, A. S. & GREENE, L. A. (1977). Nerve growth factor-induced increase in electrical excitability and acetylcholine sensitivity of a rat pheochromocytoma cell line. *Nature* **268**, 501–504.

GOULD, K. L. & HUNTER, T. (1988). Platelet-derived growth factor induces multisite phosphorylation of $pp^{60c\text{-}src}$ and increases its protein–tyrosine kinase activity. *Molecular and Cellular Biology* **8**, 3345–3356.

GREENE, L. A. & TISCHLER, A. S. (1982). PC12 pheochromocytoma cultures in neurobiological research. *Advances in Cellular Neurobiology* **3**, 373–414.

HAMILL, O. P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F. J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv* **391**, 85–100.

HASHIMOTO, S. (1988). K-252a, a potent protein kinase inhibitor, blocks nerve growth factor-induced neurite outgrowth and changes in the phosphorylation of proteins in PC12h cells. *Journal of Cell Biology* **107**, 1531–1539.

HEMPSTEAD, B. L., MARTIN-ZANCA, D., KAPLAN, D. R., PARADA, L. F. & CHAO, M. V. (1991). High-affinity NGF binding requires coexpression of the trk proto-oncogene and the low-affinity NGF receptor. *Nature* **350**, 678–683.

HIRNING, L. D., FOX, A. P., MCCLESKEY, E. W., OLIVERA, B. M., THAYER, S. A., MILLER, R. J. & TSIEN, R. W. (1988). Dominant role of N-type calcium channels in evoked release of norepinephrine from rat sympathetic neurons. *Science* **239**, 57–61.

IKEDA, S. R. (1991). Double-pulse calcium channel current facilitation in adult rat sympathetic neurones. *Journal of Physiology* **439**, 181–214.

JONES, S. W. & JACOBS, L. S. (1990). Dihydropyridine actions on calcium currents in frog sympathetic neurons. *Journal of Neuroscience* **10**, 2261–2267.

KAPLAN, D. R., HEMPSTEAD, B. L., MARTIN-ZANCA, D., CHAO, M. V. & PARADA, L. F. (1991). The trk proto-oncogene product: a signal transducing receptor for nerve growth factor. *Science* **252**, 554–558.

KAPLAN, D. R., MARTIN-ZANCA, D. & PARADA, L. F. (1991). Tyrosine phosphorylation and tyrosine kinase activity of the trk proto-oncogene product induced by NGF. *Nature* **350**, 158–160.

KAWAI, S. & HANAFUSA, H. (1971). The effects of reciprocal changes in temperature on the transformed state of cells infected with a Rous sarcoma virus mutant. *Virology* **46**, 470–479.

KOIZUMI, S., CONTRERAS, M. L., MATSUDA, Y., HAMA, T., LAZAROVICI, P. & GUROFF, G. (1988). K-252a: a specific inhibitor of the action of nerve growth factor on PC12 cells. *Journal of Neuroscience* **8**, 715–721.

KONGSAMUT, S. & MILLER, R. J. (1986). Nerve growth factor modulates the drug sensitivity of neurotransmitter release from PC12 cells. *Proceedings of the National Academy of Sciences of the USA* **83**, 2243–2247.

KYPTA, R. M., GOLDBERG, Y., ULUG, E. T. & COURTNEIDGE, S. A. (1990). Association between the PDGF receptor and members of the *src* family of tyrosine kinases. *Cell* **62**, 481–492.

LAZAROVICI, P., LEVI, B.-Z., LELKES, P. I., KOIZUMI, S., FUJITA, K., MATSUDA, Y., OZATA, K. & GUROFF, G. (1989). K-252a inhibits the increase in c-*fos* transcription and the increase in intracellular calcium produced by nerve growth factor in PC12 cells. *Journal of Neuroscience Research* **23**, 1–8.

LEVI-MONTALCINI, R. (1987). The nerve growth factor 35 years later. *Science* **237**, 1154–1162.

LEVY, J. B., DORAI, T., WANG, L. H. & BRUGGE, J. S. (1987). The structurally distinct form of $pp60^{c\text{-}src}$ detected in neuronal cells is encoded by a unique c-*src* mRNA. *Molecular and Cellular Biology* **7**, 4142–4145.

LLINÁS, R., SUGIMORI, M., LIN, J. W. & CHERKSEY, B. (1989). Blocking and isolation of a calcium channel from neurons in mammals and cephalopods utilizing a toxin fraction (FTX) from funnel-web spider poison. *Proceedings of the National Academy of Sciences of the USA* **86**, 1689–1693.

LOEB, D. M., MARAGOS, J., MARTIN-ZANCA, D., CHAO, M. V., PARADA, L. F. & GREENE, L. A. (1991). The *trk* proto-oncogene reduces NGF responsiveness in mutant NGF-nonresponsive PC12 cell lines. *Cell* **66**, 961–966.

MAHER, P. A. (1988). Nerve growth factor induces protein–tyrosine phosphorylation. *Proceedings of the National Academy of Sciences of the USA* **85**, 6788–6791.

MAHER, P. A. (1989). Role of protein tyrosine phosphorylation in the NGF response. *Journal of Neuroscience Research* **24**, 29–37.

MANDEL, G., COOPERMAN, S. S., MAUE, R. A., GOODMAN, R. H. & BREHM, P. (1988). Selective induction of brain type II Na^+ channels by nerve growth factor. *Proceedings of the National Academy of Sciences of the USA* **85**, 924–928.

MARTINEZ, R., MATHEY-PREVOT, B., BERNARDS, A. & BALTIMORE, D. (1987). Neuronal $pp60^{c\text{-}src}$ contains a six amino acid insertion relative to its non-neuronal counterpart. *Science* **237**, 411–415.

MILLER, R. J. (1990). Receptor-mediated regulation of calcium channels and neurotransmitter release. *FASEB Journal* **4**, 3291–3298.

MINTZ, I. M., VENEMA, V. J., SWIDEREK, K. M., LEE, T. D., BEAN, B. P. & ADAMS, M. E. (1992). P-type calcium channels blocked by the spider toxin ω-Aga-IVA. *Nature* **355**, 827–829.

MORGAN, J. I. & CURRAN, T. (1986). Role of ion flux in the control of c-fos expression. *Nature* **322**, 552–555.

NOWYCKY, M. C., FOX, A. P. & TSIEN, R. W. (1985). Three types of neuronal calcium channel with different calcium channel agonist selectivity. *Nature* **316**, 440–443.

OLIVERA, B. M., RIVIER, J., CLARK, C., RAMILO, C. A., CORPUZ, G. P., ABOGADIE, Fe C., MENA, E. E., WOODWARD, S. R., HILLYARD, D. R. & CRUZ, L. J. (1990). Diversity of *Conus* neuropeptides. *Science* **249**, 257–263.

PLUMMER, M. R., LOGOTHETIS, D. E. & HESS, P. (1989). Elementary properties and pharmacological sensitivities of calcium channels in mammalian peripheral neurons. *Neuron* **2**, 1453–1463.

QUI, M.-S. & GREEN, S. H. (1991). NGF and EGF rapidly activate $p21^{ras}$ in PC12 cells by distinct, convergent pathways involving tyrosine phosphorylation. *Neuron* **7**, 937–946.

RALSTON, R. & BISHOP, J. M. (1985). The product of the proto-oncogene c-*src* is modified during the cellular response to platelet derived growth factor. *Proceedings of the National Academy of Sciences of the USA* **82**, 7845–7849.

RAUSCH, D. M., DICKENS, G., DOLL, S., FUJITA, K., KOIZUMI, S., RUDKIN, B. B., TOCCO, M., EIDEN, L. E. & GUROFF, G. (1989). Differentiation of PC12 cells with v-*src*: comparison with nerve growth factor. *Journal of Neuroscience Research* **24**, 49–58.

RAUSCH, D. M., LEWIS, D. L., BARKER, J. L. & EIDEN, L. E. (1990). Functional expression of dihydropyridine-insensitive calcium channels during PC12 cell differentiation by nerve growth factor (NGF), oncogenic ras, or src tyrosine kinase. *Cellular and Molecular Neurobiology* **10**, 237–255.

RUDY, B., KIRSCHENBAUM, B., RUKENSTEIN, A. & GREENE, L. A. (1987). Nerve growth factor increases the number of functional Na channels and induces TTX-resistant Na channel in PC12 pheochromocytoma cells. *Journal of Neuroscience* **7**, 1613–1625.

SANGUINETTI, M. D. & KASS, R. S. (1984). Voltage-dependent block of calcium channel current by dihydropyridine calcium channel antagonists. *Circulation Research* **55**, 336–348.

SORGE, L. K., LEVY, B. T. & MANESS, P. F. (1948). $pp60^{c\text{-}src}$ is developmentally regulated in the neural retina. *Cell* **36**, 249–257.

STANLEY, E. F. & GOPING, G. (1991). Characterization of a calcium current in a vertebrate cholinergic presynaptic nerve terminal. *Journal of Neuroscience* **11**, 985–993.

STREIT, J. & LUX, H. D. (1987). Voltage-dependent calcium currents in PC12 growth cones and cells during NGF-induced cell growth. *Pflügers Archiv* **408**, 634–641.

TAKAHASHI, M., TSUKUI, H. & HATANAKA, H. (1985). Neuronal differentiation of Ca^{2+} channel by nerve growth factor. *Brain Research* **341**, 381–384.

THOENEN, H. (1991). The changing scene of neurotrophic factors. *Trends in Neurosciences* **14**, 165–170.

THOMAS, S. M., HAYES, M., D'ARCANGELO, G., ARMSTRONG, R. C., MEYER, B. E., ZILBERSTEIN, A., BRUGGE, J. S. & HALEGOUA, S. (1991). Induction of neurite outgrowth by v-*src* mimics critical aspects of nerve growth factor-induced differentiation. *Molecular and Cellular Biology* **11**, 4739–4750.

USOWICZ, M. M., PORZIG, H., BECKER, C. & REUTER, H. (1990). Differential expression by nerve growth factor of two types of Ca^{2+} channels in rat phaeochromocytoma cell lines. *Journal of Physiology* **426**, 95–116.