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Expression and Subunit Interaction of Voltage-Dependent Ca^{2+} Channels in PC12 Cells

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Nerve growth factor (NGF)-induced differentiation in PC12 cells is accompanied by changes in the expression of voltage-dependent Ca^{2+} channels. Ca^{2+} channels are multimeric complexes composed of at least three subunits (α_1, β, and $\alpha_2\delta$) and are involved in neuronal migration, gene expression, and neurotransmitter release. Although attempts have been undertaken to elucidate NGF regulation of Ca^{2+} channel expression, the changes in subunit composition of these channels during differentiation still remain uncertain. In the present study, patch-clamp recordings show that in addition to the previously documented L-type and N-type Ca^{2+} currents, undifferentiated PC12 cells also express an ω-agatoxin-IVA-sensitive (P/Q-type) component. In addition, the corresponding mRNA encoding the pore-forming α_1 subunits for these channels (C, B, and A, respectively) was detected. Likewise, mRNA for three distinct auxiliary β subunits (1, 2, 3) were also found, β_3 protein being dominantly expressed. Immunoprecipitation experiments show that the N-type Ca^{2+} channel is associated with either a β_2 or β_3 subunit and that NGF increases the channel expression without affecting its β subunit association. These results (1) indicate that the diversity of Ca^{2+} currents in PC12 cells arise from the expression of three distinct α_1 and three different β subunit genes; (2) support a model for heterogenous β subunit association of the N-type Ca^{2+} channel in a single cell type; and (3) suggest that the regulation of the N-type Ca^{2+} channel during NGF-mediated differentiation involves an increase in the number of functional channels with no apparent changes in subunit composition.

Key words: calcium channels; α_{1B} subunit; β subunit; PC12; nerve growth factor; P/Q-type; N-type; ω-agatoxin-IVA; ω-conotoxin GVIA

Voltage-dependent Ca^{2+} channels play an important role in regulating cellular Ca^{2+} concentration in excitable cells (Tsien et al., 1995). These Ca^{2+} channels not only are central in controlling neurotransmitter release, excitation–contraction coupling, and excitation–secretion coupling, they are also involved in gene expression and neuronal migration (Beam et al., 1992; Komuro and Rakic, 1992; Ghosh et al., 1994; Dunlap et al., 1995). Several voltage-dependent Ca^{2+} channels have been identified and carry out diverse functions: T-, L-, N-, P-, Q-, and R-type (Llinas et al., 1992; Zhang et al., 1993; Basarsky et al., 1994). The structure of the Ca^{2+} channels was elucidated with the purification of skeletal muscle L-type and brain N-type channel, which are composed of at least three protein subunits (α_1, β, and $\alpha_2\delta$) (Leung et al., 1987; Takahashi et al., 1987; McEnery et al., 1991; Witcher et al., 1993).

The α_1 subunit forms the Ca^{2+} channel pore and binds Ca^{2+} channel blockers (Tanabe et al., 1987; Hockerman et al., 1995). Six different α_1 genes have been identified thus far (S, A, B, C, D, and E), and with the exception of S, all are expressed in the nervous system (Birnbaumer et al., 1994). The class B α_1 gene encodes for the N-type Ca^{2+} channel (Dubel et al., 1992; Williams et al., 1992; Witcher et al., 1993), whereas the product of class A α_1 gene is a component of the P/Q-type Ca^{2+} channel (Mori et al., 1991; Liu et al., 1996).

The β subunit directly associates with the α_1 subunit (De Waard et al., 1994; Pragnell et al., 1994) and is essential for normal function and localization of the α_1 subunit (Castellano et al., 1993a,b; Olcese et al., 1994; Chien et al., 1995). Similar to the multitude of the α_1 subunits, at least four different β genes (1, 2, 3, and 4) have been identified (Birnbaumer et al., 1994).

Rat PC12 pheochromocytoma cell line has been a model system to study neuronal differentiation (Greene and Tischler, 1976; Shafer and Atchison, 1991; Chao, 1992). PC12 cells are chromaffin-like cells that begin to resemble sympathetic neurons when exposed to nerve growth factor (NGF). Previously, functional N- and L-type Ca^{2+} channels were detected in PC12 cells (Usowicz et al., 1990; Avidor et al., 1994). In addition, it has been shown that NGF increases mRNA expression of several Ca^{2+} channel α_1 subunits (Lievano et al., 1994). Although in the highly heterogeneous brain, it has been reported recently that β_3, β_4, and β_{1b} subunits all are capable of associating with the α_{1B} subunit to form distinct brain N-type channels (Scott et al., 1996), it is not clear whether different β subunits can associate with the N-type channels in a single cell type. Furthermore, it is uncertain whether there is any change of the subunit composition of the Ca^{2+} channels during NGF-induced differentiation. To address these questions, we have investigated, at the molecular level, the expression of Ca^{2+} channel α_1 and β subunits in PC12 cells. In

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addition, we have characterized the β subunit composition of the PC12 N-type Ca^{2+} channel during NGF-induced differentiation.

MATERIALS AND METHODS

Cell culture. PC12 cells (American Type Culture Collection, Rockville, MD) were grown on rat collagen type I (Collaborative Biomedical Products, Bedford, MA) coated dishes in RPMI 1640 medium supplemented with 5% horse serum and 10% fetal calf serum in a 5% CO_2 air-humidified atmosphere. The medium was changed every 3 d, and the cells were subcultured approximately every 10 d in a ratio of 1:6. Cells were plated 1 d before the NGF (Promega, Madison, WI) addition. NGF (50 ng/ml) was added to the culture medium for a 3 d period to induce PC12 differentiation.

Patch-clamp experiments. For electrophysiological recording, undifferentiated PC12 cells were plated on poly-L-lysine-coated glass coverslips (10 × 25 mm) and grown in RPMI 1640 medium supplemented with 5% fetal bovine serum, 1% L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. After 3–5 d in culture, the cells were subjected to the standard whole-cell patch-clamp technique (Hamill et al., 1981) using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). Current signals were filtered at 1 kHz (internal four-pole Bessel filter), digitized at 50 kHz, and analyzed with pClamp software (Axon Instruments). Patch pipettes were pulled from borosilicate glass capillaries (KIMAX-51; Kimble Division, Owens-Illinois, Toledo, OH) on a horizontal puller (Sutter Instrument, Novato, CA), and pipette tips were fire-polished with a microforge (Narishige, Tokyo, Japan). Typical electrode resistances were 2–5 MΩ. The bath solution contained (in mM): 20 $BaCl_2$, 125 tetraethylammonium chloride (TEA-Cl), 10 HEPES, and 10 glucose, pH 7.3. The internal (patch-pipette) solution consisted of (in mM): 130 CsCl, 2 $MgCl_2$, 11 EGTA, 20 HEPES, 2 Na_2ATP, 0.1 GTP, and 10 glucose, pH 7.3. After establishing the whole-cell mode, capacitative transients were canceled with the amplifier. Currents were obtained by applying 150 msec test pulses every 30 sec from a holding potential of −90 mV. Leakage currents were usually less than −20 pA at holding potential. Data were leak-subtracted on line by a standard P/4 procedure. ω-agatoxin-IVA (ω-Aga-IVA, Peptides International, Louisville, KY) was prepared as a 10 μM stock in distilled water, and aliquots were stored at −20°C. A fresh aliquot was diluted in the bath solution for each experiment to give a final concentration of 200 nM. All experiments were performed at 20–22°C.

Membrane preparations and immunoblot analysis. Crude rat brain membranes and cardiac microsome membranes were prepared as detailed elsewhere (Sakamoto and Campbell, 1991). PC12 cell membranes were prepared as follows. Preconfluent PC12 cells were washed with PBS and harvested with a cell scraper. Cells were collected by centrifugation at 500 × *g* and homogenized in buffer A (5 mM HEPES, pH 7.4, 0.3 M sucrose, 0.1 mM phenylmethylsulfonyl fluoride, 0.75 mM benzamidine, 0.6 μg/ml pepstatin A, 0.5 μg/ml aprotinin, 0.5 μg/ml leupeptin, 0.01 mg/ml lysozyme). Cell membranes were collected by centrifugation at 135,000 × *g* for 30 min, resuspended in buffer A, and stored at −80°C. PC12 and rat tissue membranes (100–250 μg of protein/lane) were resolved on 3–12% gradient SDS–polyacrylamide gels and transferred to nitrocellulose. ECL detection system (Amersham, Arlington Heights, IL) was used according to the manufacturer's instructions.

Total RNA isolation, RT-PCR, and sequencing. Total RNA was isolated by homogenization in RNAzol according to the protocol of Cinna/Biotecx (Friendswood, TX) followed by chloroform extraction. The RNA was stored at −80°C as precipitates in 70% ethanol. To eliminate any possible contamination by genomic DNA, the total RNA was treated with DNase RQ 1 (Promega, Madison, WI) for 30 min at 37°C before reverse transcription PCR (RT-PCR).

cDNA was synthesized from 2 μg total RNA by 400 U Moloney murine leukemia virus reverse transcriptase in a total volume of 30 μl. Typically, 4 μl of cDNA was used in 50–100 μl PCR. The PCR reactions were usually carried out for 32–36 cycles of 94°C 1 min, 56°C 1 min, and 72°C 1 min. The primers used are as follows: rat β_{1b} forward 5′-TCCAGGGACCCTACCTTGTTTCC-3′ (1391–1413, rat β_{1b} GenBank no. X61394); rat β_{1b} reverse 5′-CCTCCAGCTCATTCTTATTG-CGC-3′ (1806–1828); rat β_2 forward 5′-TCGGATCCGAAGAAGAA-CCTTGTCTGG-3′ (1759–1777, GenBank no. 80545); rat β_2 reverse 5′-TCGAATTCAGTAGCGATCCTTAGATTTATGC-3′ (2087–2110); rat β_3 forward 5′-GTGGTGTTGGATGCTGAC-3′ (892–909, GenBank no. M88751); rat β_3 reverse 5′-ATTGTGGTCATGCTCCGA-3′ (1483–1500); rat β_4 forward 5′-TTGGATCCACAGCTCTCTCACCGTA-TCC-3′ (1457–1479, GenBank no. L02315), rat β_4 reverse 5′-TAGGAT-CCAGGGTAGTGATCTCGGCTG-3′ (1639–1664); rat α_{1A} forward 5′-CCAGTCTGTGGAGATGAGAGAAATGGG-3′ (6042–6068, GenBank no. M64373); rat α_{1A} reverse 5′-TTTGGAGGGCAGGTCACCCG-ATTG-3′ (6412–6435); rat α_{1B} forward 5′-GCCGTCTCAGCCGCG-GCCTTTCT-3′ (6668–6690, GenBank no. M92905); α_{1B} reverse 5′-CAAAGGTGAGTGTATCCTCAGGC-3′ (6810–6832); α_{1C} forward 5′-GGAAGGATCCGAGCGAAAGAAGCTGGC-3′ (3064–3090 in rat α_{1C} rbc-I, GenBank no. M67516); α_{1C} reverse 5′-AGGTGAATTCGG-CCCACAGGCATCTCG-3′ (3307–3333 rat α_{1C} rbc-I); α_{1D-1} forward 5′-GGAGAGGAGGGCAAACGAAACACTAGC-3′ (1892–1918, GenBank no. M57682); α_{1D-1} reverse 5′-CGTACACACCGGAACACAGAGA-CGC-3′ (2364–2388); α_{1D-2} forward 5′-GTGCCCTGCACACAGTAGGC-GC-3′ (485–506); α_{1D-2} reverse 5′-GGCACGGGCAGGTCGGCT-GTTAG-3′ (816–838); α_{1E} forward 5′-TCGGATCCAAATGTGAAG-AGGAGCGTATC-3′ (2569–2597, GenBank no. L15453), α_{1E} reverse 5′-AGTCAAGCTTCACGTCAGGGATGGCGACTG-3′ (3262–3291).

The β_1 and β_3 RT-PCR products from PC12 total RNA were directly sequenced by the dideoxy chain termination method using total PCR products as sequencing templates (Barnard et al., 1994). The β_1 PCR product was also subcloned into pBluescript, and its sequence was confirmed by cycle sequencing from both directions. All others were subcloned into pGEX 2T or pBluescript and sequenced by the dideoxy chain termination method according to United States Biochemicals (Cleveland, Ohio).

Enrichment of β_2 subunit using β_2 antibody affinity column. Affinity-purified rabbit 143 (β_2) antibody was coupled to Hydrazide Avidgel AX according to the manufacturer's instructions (Unisyn Technologies, San Diego, CA). PC12 membranes (25 mg) were solubilized for 1 hr at 4°C in solubilization buffer (1% digitonin, 1 M NaCl, 10 mM HEPES, pH 7.4, 0.75 mM benzamidine, 0.6 μg/ml pepstatin A, 0.5 μg/ml aprotinin, 0.5 μg/ml leupeptin). The solubilized materials were sedimented by centrifugation in a Beckman TL 100 ultracentrifuge for 10 min at 400,000 × *g*. Then the supernatant was diluted eightfold with 10 mM HEPES containing the above-mentioned protease inhibitors. The diluted material (50 ml) was applied to the antibody affinity column. After washing the column extensively with 50 ml of buffer B (10 mM HEPES, pH 7.4, 0.1% digitonin, 100 mM NaCl), the column was eluted with glycine buffer (50 mM glycine, pH 2.5, 100 mM NaCl, 0.1% digitonin) containing the protease inhibitors. A 125 μl volume of 2 M Tris, pH 8.0, was added to every milliliter of eluate immediately after the elution. The eluted material was concentrated with Centricon-100 (Amicon, Beverly, WA) to 1–2 ml, and 0.1 ml per lane was loaded onto SDS-polyacrylamide gel for analysis.

Immunoprecipitation of N-type Ca^{2+} channels. PC12 cell membranes were solubilized and diluted as described above. The diluted extract was labeled with 50 pM [^{125}I]ω-conotoxin GVIA (ω-CgTX) (Amersham, Arlington Heights, IL) for 1 hr at room temperature. Subsequently, aliquots (1 ml) of the labeled extract were incubated overnight at 4°C with 50 μl antibody–protein G Sepharose beads (Pharmacia, Piscataway, NJ). The beads were extensively washed, and total binding was quantified by γ counting. Nonspecific binding was determined by the addition of 1000-fold excess unlabeled ω-CgTX (Bachem, Torrance, CA) before the addition of the labeled toxin. The nonspecific counts were subtracted from total counts to give specific counts. Each sample point represents the average of triplicates.

RESULTS

Detection of P/Q-type Ca^{2+} channels

Ca^{2+} currents in undifferentiated PC12 cells consist of dihydropyridine (L-type) and ω-CgTX (N-type)-sensitive components (Usowicz et al., 1990), as well as a component that is resistant to both pharmacological agents (Rane and Pollock, 1994). Likewise, it is known that undifferentiated PC12 cells resemble normal chromaffin cells (Greene and Tischler, 1976), which in addition to N- and L-type Ca^{2+} currents, possess a component sensitive to the P/Q-type Ca^{2+} channel blocker ω-Aga-IVA (Artalejo et al., 1994; Gandia et al., 1995). To investigate the presence of P/Q-type functional Ca^{2+} channels, ω-Aga-IVA was applied to PC12 cells, and whole-cell Ba^{2+} inward currents were recorded. As described previously (Randall and Tsien, 1995), although some of the ω-Aga-IVA sensitive current is inhibited quickly, blockage increases progressively until a stable plateau is reached ~5 min after toxin application. Therefore, to ensure a saturating degree of

inhibition and to minimize the impact of the rundown on Ca^{2+} current, cells were preincubated for 30 min with 200 nM ω-Aga-IVA before initiating whole-cell recording. This concentration of the toxin produces maximal block of P/Q-type Ca^{2+} currents (Pearson et al., 1995).

The average amplitude of Ba^{2+} currents (I_{Ba}) in the control condition and after the preincubation with ω-Aga-IVA is plotted as a function of membrane potential (V_m) in Figure 1*A*. In both cases, the peak current increases steeply over the range between −20 and 10 mV, reaching a maximum value at +20 mV. The shape of these current–voltage (*I–V*) relationships suggests that PC12 cells exhibit a large component of high-voltage-activated Ca^{2+} channels, whereas low-voltage-activated Ca^{2+} channels are virtually absent. As illustrated in the *I–V* curve indicated by filled circles, the preincubation with ω-Aga-IVA induced a decrease in peak current amplitude at all potentials examined.

Typical Ba^{2+} currents from two distinct PC12 cells are shown in Figure 1*B*, one cell subjected to a sham preincubation (*upper trace*) and the other preincubated with ω-Aga-IVA (*lower trace*), as described above. Under the conditions of these experiments, test pulses elicited a rapidly activating inward sustained current, the average amplitude of which was significantly reduced in the toxin-preincubated cells. On average, I_{Ba} was inhibited ~33% from a mean control value of −109 ± 9 pA to −73 ± 17 pA on treatment with ω-Aga-IVA (Fig. 1*C*).

To gain insight into the ω-Aga-IVA sensitive component of the whole-cell Ca^{2+} channel current in PC12 cells, we recorded Ba^{2+} currents immediately before and after 1–6 min of exposure to 200 nM ω-Aga-IVA in individual cells. The results of these experiments indicated that PC12 cells differ markedly in their response to the toxin. We found that ω-Aga-IVA was capable of blocking a fraction of Ca^{2+} channel currents in about one-third of the investigated cells (n = 16) over a 6 min period. The curve indicated by filled circles in Figure 2*A* shows the inhibition of ω-Aga-IVA-sensitive current, as evidenced by a decrease in normalized total current from 1.0 to 0.62 ± 0.07 after 6 min toxin exposure. Note that under our recording conditions, I_{Ba} amplitude in control cells (*open circles*) decreased from 1.0 to 0.91 ± 0.02 with no detectable changes in waveform. A straight line provided a close fit to these rundown data, which were given to demonstrate the specific inhibitory effect of ω-Aga-IVA.

Upper traces in Figure 2*B* show representative currents from one ω-Aga-IVA responsive cell immediately before (*b*) and 6 min after (*a*) the application of the toxin. Subtraction of the current remained after exposure to the toxin from the current before treatment enabled us to dissect out the blocked component, as illustrated in lower trace (*b-a*). According to this analysis, ω-Aga-IVA-sensitive current averaged 51 ± 10 pA in amplitude and accounted for 32 ± 2% of the total I_{Ba}. In addition, the inactivation phase of these subtracted currents were fitted with a single exponential equation of the form: $A\exp(-t/\tau) + C$, where A is the initial amplitude (pA), t is time (msec), τ is the time constant for inactivation, and C is a constant. The application of the toxin resulted in the inhibition of a current component that exhibited a degree of decay of 17.5 ± 3.1% over the 150 msec voltage step and an average time constant (τ) of 88.7 ± 9.4 msec.

mRNA expression of multiple α_1 and β subunits in PC12 cells

Because several different types of Ca^{2+} channels are present in PC12 cells, these cells should express mRNA of various α_1 subunits. RT-PCR was performed on PC12 total RNA to investigate

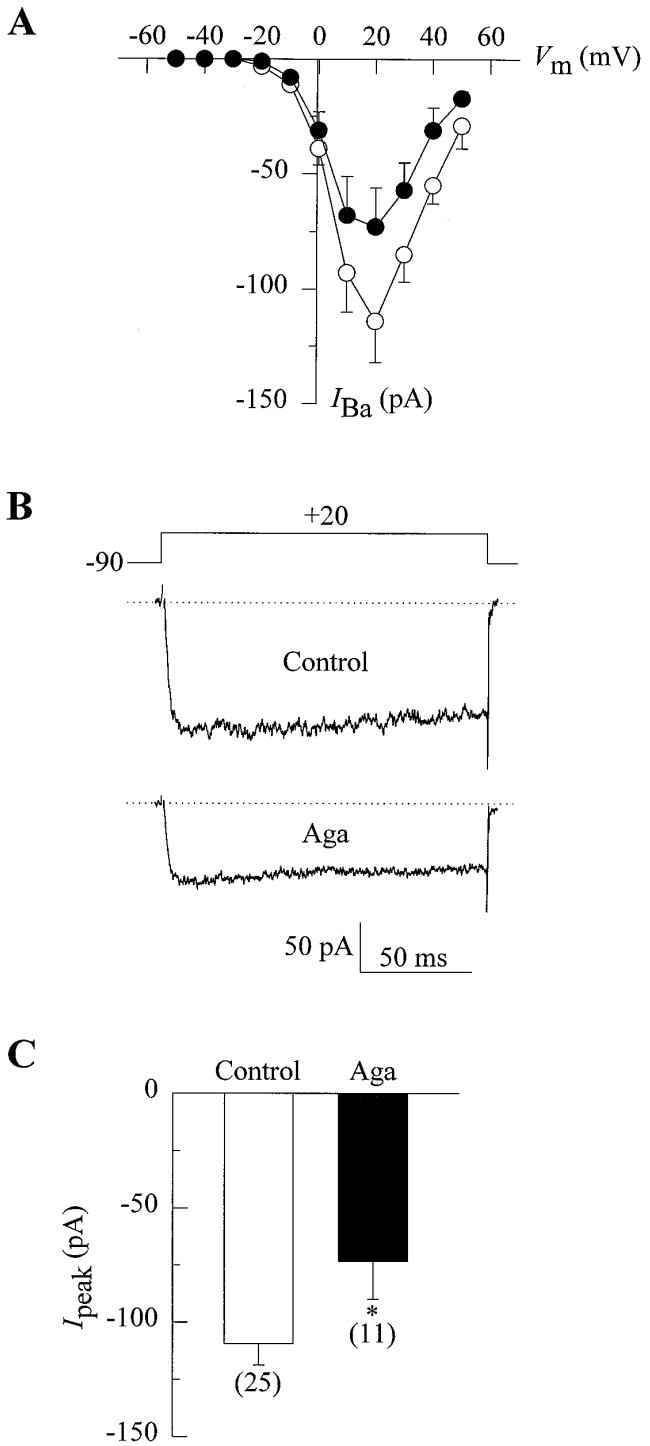


Figure 1. Voltage dependence of Ba^{2+} current in PC12 cells and its inhibition by ω-Aga-IVA. *A*, Peak current–voltage relationships obtained from two groups of cells after 30 min incubation in the absence (*open circles*) or presence of 200 nM ω-Aga-IVA (*filled circles*). The currents were recorded in response to 150 msec depolarization from a holding potential of −90 mV with 10 mV increase in the pulse amplitude per step. *Symbols* represent mean peak current values of sham preincubated (n = 9) and toxin-treated cells (n = 11). *B*, Illustrative traces of peak inward current from a control (*upper trace*) and an ω-Aga-IVA-treated (*lower trace*) cell. The voltage protocol is shown *above* the traces, and the *dotted line* represents baseline current. *C*, Comparison of peak current amplitudes at +20 mV in both control and ω-Aga-IVA-treated cells. Data are given as mean ± SE, and the number of recorded cells is indicated in *parentheses*. *Asterisks* denote significant differences ($p < 0.05$).

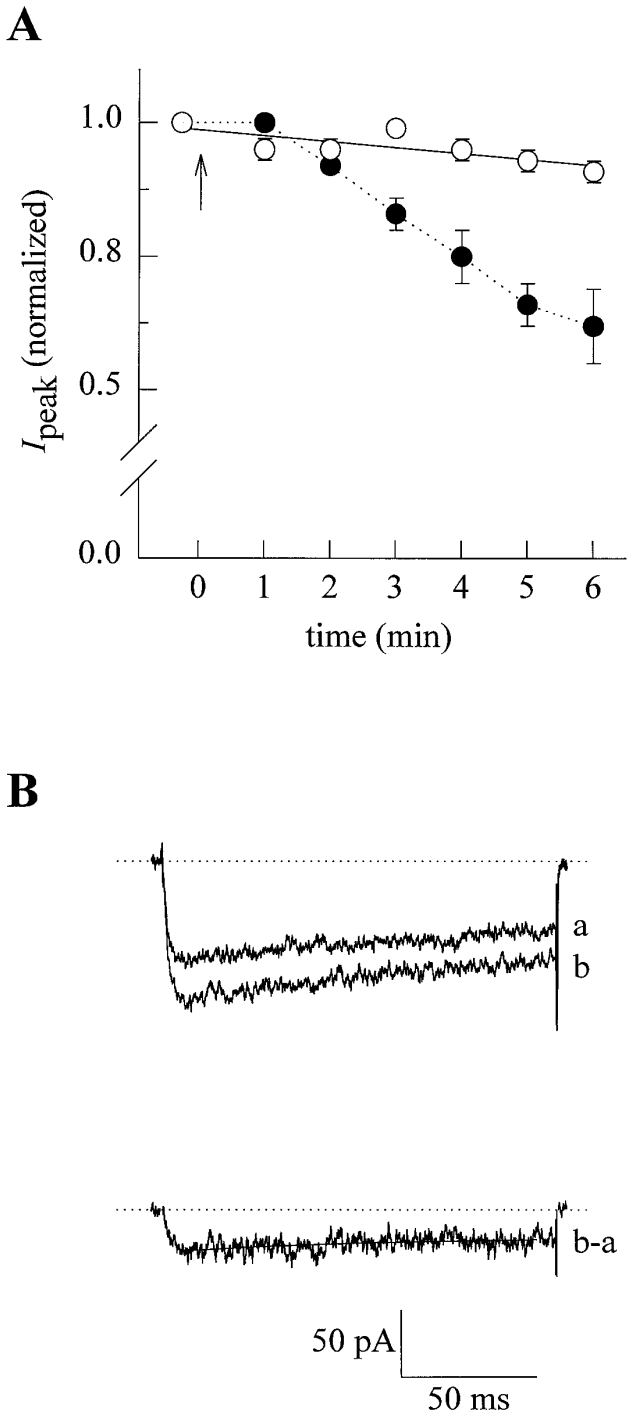


Figure 2. Properties of the ω-Aga-IVA-sensitive Ca^{2+} channel current in PC12 cells. *A*, Plot of peak I_{Ba} versus time in which recordings were sequentially taken before and after ω-Aga-IVA application. *Filled circles* represent average current blocked by the toxin ($n = 5$). Currents were obtained by applying 150 msec steps from −90 to +20 mV, and peak currents were normalized by the values observed before toxin application. *Arrow* indicates ω-Aga-IVA addition. *Open circles* denote time-dependent changes in the amplitude of the current (rundown) in control cells, and the *straight line* represents the best fit to the data. *B*, Representative superimposed traces of Ba^{2+} currents taken from one of the cells indicated in *A*, before (*b*) and 6 min after exposure to 200 nM ω-Aga-IVA (*a*). *Lower trace* exemplifies the currents (*b-a*) obtained by subtracting the current in the absence and in the presence of the toxin and represents the ω-Aga-IVA-sensitive component. The inactivation phase of these currents was fitted with a single exponential (*solid line*).

the α_1 and β subunit genes expressed in these cells. PCR of total RNA, without reverse transcription, was performed as a negative control in parallel with RT-PCR (data not shown). RT-PCR with three pairs of α_1 subunit primers amplified PC12 cDNA of the predicted sizes (Fig. 3*A*). Sequencing of these PCR products confirmed the expression of three α_1 subunit genes (*A*, *B*, *C*) in PC12 cells (Fig. 3*B*). The sequences of the RT-PCR products are identical to nucleotide 6259–6386 of rat α_{1A} (GenBank accession no. M64373), nucleotide 6685–6835 of rat α_{1B} (GenBank accession no. M92905), and nucleotide 3162–3316 of a splice variant rbc-I of rat α_{1C} (GenBank accession no. M67516) (Snutch et al., 1991), respectively. These data are in agreement with the expression of L-type (α_{1C}), N-type (α_{1B}), and P/Q-type (α_{1A}) Ca^{2+} channels. α_{1D} and α_{1E} subunits were not detected using RT-PCR either because of their absence or very low level of expression.

Because Ca^{2+} channel β subunits have two highly homologous domains, two pairs of primers that specifically amplified a portion of β_2 or β_3 control cDNA in PCR reactions were used to amplify PC12 cDNA (Fig. 4*A*). PCR of PC12 cDNA with these primers amplified DNA fragments of identical molecular weights as the products of control β cDNA. The sequence of β_2 RT-PCR product from PC12 cells is 100% identical to nucleotide 1759–1990 of rat β_2 (GenBank accession no. M80545) in a 232-nucleotide stretch. The β_3 RT-PCR product is 100% identical to nucleotide 984–1122 of rat β_3 (GenBank accession no. M88751) in the 143 bases sequenced. The β_1 primers efficiently amplify control β_1 cDNA, although they also weakly amplify control β_4 cDNA. However, no β_4 sequence was identified in sequencing 14 subcloned β_1 PCR products. The sequence of the β_1 RT-PCR product is 99% identical to rat β_{1b} (GenBank accession no. X61394) (Pragnell et al., 1991) with only three nucleotide differences between the PCR product and the rat β_{1b} sequence in a stretch of 435 nucleotides (Fig. 4*B*). Two of the three single nucleotide changes (T1537→C and T1595→C) result in W492R and V511A amino acid changes. The last two different nucleotides (C188, C202) in PC12 β_1 are the same as the corresponding nucleotides in human β_1 sequence (Collin et al., 1993). Therefore, PC12 cells express a β_1 isoform that is very similar to the β_{1b} isoform expressed in brain. β_4 was not detected using either the β_1 primers or a pair of β_4 primers, suggesting an absence or extremely low level of expression. These results demonstrate that PC12 cells express mRNA that encode for β_1, β_2, and β_3 subunits.

Association of different β subunits with the α_{1B} subunit

The N-type Ca^{2+} channel represents the most abundant Ca^{2+} channel in PC12 cells (Usowicz et al., 1990) and has been shown to be heterogeneous in its β subunit composition in brain (Scott et al., 1996). Because several β subunits are expressed in PC12 cells, it is important to identify the β subunit(s) associated with the N-type channel. We first examined the protein expression of these β subunits in PC12 cells. Four β subtype-specific antibodies were produced against β_{1b}, β_2, β_3, and β_4 subunits, respectively (Liu et al., 1996; Scott et al., 1996). These polyclonal β subunit-specific antibodies recognize a β_1 subunit of 78 and 80 kDa, a β_3 subunit of 58 kDa, and a β_4 subunit of 59 and 55 kDa in crude rat brain membranes, and β_2 subunits of 87, 74, and 70 kDa in rat cardiac microsome membranes (Fig. 5*A*). The β_2 antibody only detected the 74 kDa β_2 in rat brain (data not shown). These results demonstrate that the four β subunit-specific antibodies recognize native rat β subunit proteins. There is also evidence that at least four β subunits are present in rat whole brain (β_1, β_2, β_3, and β_4) and that β_2 subunit is present in rat cardiac tissue. The multiple

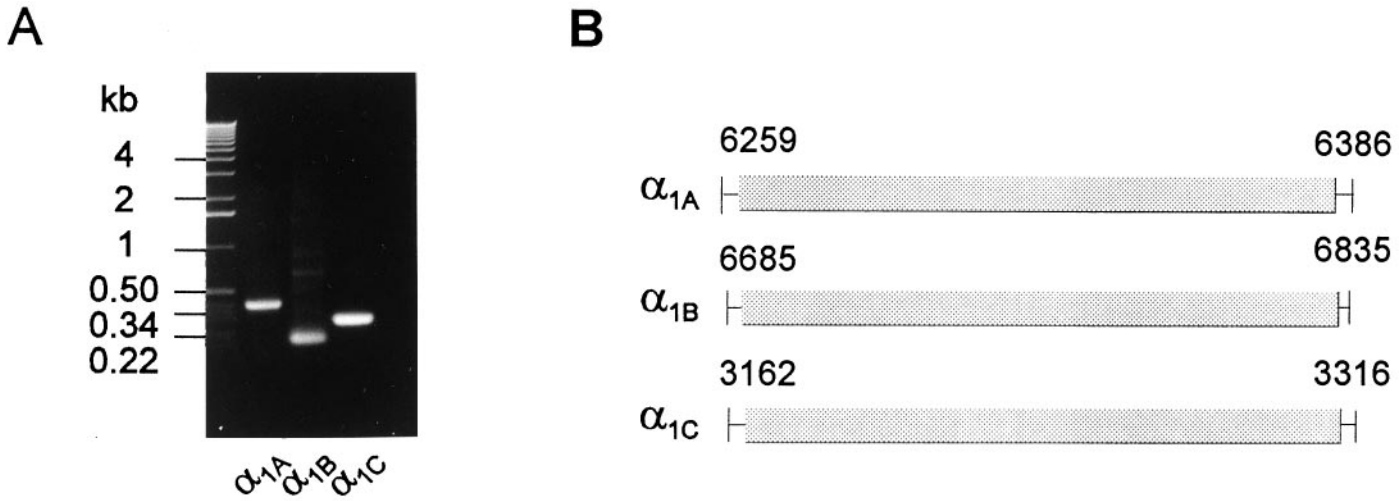


Figure 3. mRNA expression of three Ca^{2+} channel α_1 subunits in PC12 cells. *A*, RT-PCR from PC12 cell total RNA with α_{1A}, α_{1B}, and α_{1C} primers. The PCR products were separated on a 1% agarose gel. The molecular weight standards are on the *left*. *B*, Schematic representation of the regions of published rat α_{1A}, α_{1B}, and α_{1C} rbc-I sequences, which are identical to the sequences of PC12 α_{1A}, α_{1B}, and α_{1C} RT-PCR products, respectively. The *numbers* stand for the nucleotide positions in the published rat sequences.

bands seen in immunoblot of β_1, β_2, and β_4 subunit may be attributable to post-translational modification or multiple β subunit isoforms (Collin et al., 1993; Chien et al., 1995). These same four polyclonal β antibodies were used to detect the β subunits in PC12 cells. β_3 subunit was easily detected from crude membranes of both undifferentiated and differentiated PC12 cells, whereas no other β subunits were ever detected (Fig. 5*B*). This suggests a dominant expression of β_3 subunit in PC12 cells. An ~10-fold enrichment by a β_2 antibody affinity column was required to visualize the β_2 subunit by immunoblot analysis. As shown in Figure 5*B*, a 70 kDa β_2 subunit appeared in the eluate of the β_2 antibody affinity column. The size of this β_2 subunit is comparable to the post-translational modified form of this subunit expressed in human embryonic kidney cells (Chien et al., 1995).

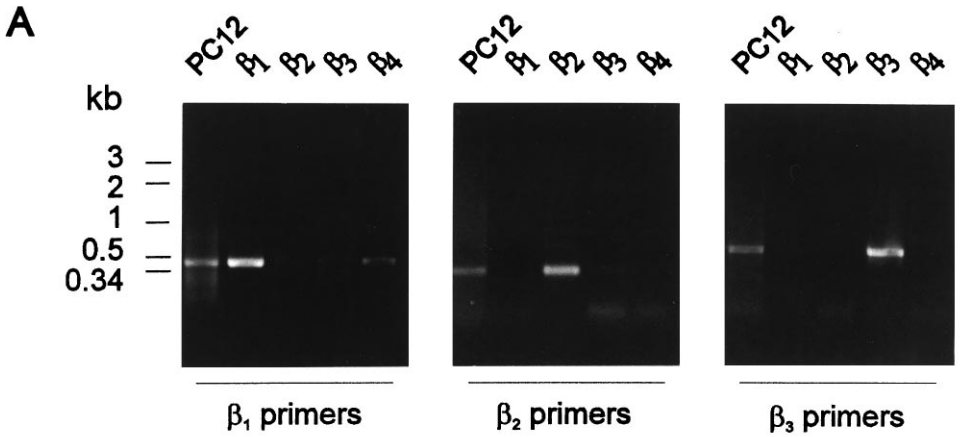
Because expression of the α_{1B} subunit of the N-type Ca^{2+} channel is upregulated by NGF (Lievano et al., 1994), we investigated whether the β subunit composition of this channel is modified by NGF treatment. To examine the interaction between α_{1B} and β subunits in PC12 cells, the above-mentioned four β subunit-specific antibodies and polyclonal antibodies against α_{1B} GST fusion protein (Y006) were used for immunoprecipitation of [^{125}I]ω-CgTX-labeled N-type Ca^{2+} channels (Fig. 5*C*). Polyclonal antibodies against β_3 immunoprecipitated 74 ± 4% (n = 3) of [^{125}I]ω-CgTX labeled receptors from undifferentiated PC12 membrane extracts, followed by β_2 antibody 22 ± 3% (n = 3). No immunoprecipitation was detected with protein G beads alone used as a negative control. In addition, polyclonal antibodies against β_{1b} and β_4 subunits were unable to immunoprecipitate significant amounts of labeled receptors from undifferentiated PC12 membrane extracts (<10%), which may result from either the low level of expression or the sensitivity of the methods. Therefore, the α_{1B} subunits are associated with either β_3 or β_2 subunits in the N-type Ca^{2+} channels of undifferentiated PC12 cells.

NGF treatment induced striking morphological changes in PC12 cells. After 3 d of NGF treatment, >70% of PC12 cells in culture had grown neurites longer than the cell diameter (data not shown). Lievano et al. (1994) has reported that the upregulation of the α_{1B} mRNA expression reached the maximum plateau as early as day 1 of NGF application. Therefore, the association of the different β subunits with the α_{1B} subunit was examined in the 3 d NGF-treated PC12 cells (Fig. 5*C*). The total expression of α_{1B} subunits per milligram of membrane protein was increased ~59%. Notably, the polyclonal antibody against β_3 immunoprecipitated 74 ± 2% (n = 3) of [^{125}I]ω-CgTX-labeled receptors, followed by β_2 antibody 21 ± 3% (n = 3). Again, polyclonal antibodies against β_{1b} and β_4 subunits immunoprecipitated negligible amounts of [^{125}I]ω-CgTX-labeled receptors. Therefore, the same proportion of α_{1B} subunits in both undifferentiated and differentiated PC12 cells is associated with either the β_3 or β_2 subunits, although the expression of N-type Ca^{2+} channels increased significantly after NGF treatment.

DISCUSSION

In this paper, we provide evidence for the presence of ω-Aga-IVA-sensitive Ca^{2+} channels in undifferentiated PC12 cells. This component of the whole-cell Ca^{2+} current has not been reported previously in this cell line. Our results confirm previous reports (Usowicz et al., 1990; Rane and Pollock, 1994) that describe global Ca^{2+} channel current in undifferentiated PC12 cells as consisting of a large component of high-voltage-activated Ca^{2+} current and no low-voltage-activated Ca^{2+} current. In addition, preincubation experiments indicated that exposure to nanomolar concentrations of ω-Aga-IVA resulted in a significant decrease in average peak I_{Ba} amplitude (Fig. 1). This inhibitory effect was confirmed when I_{Ba} was sequentially recorded from patch-clamped cells before and after toxin application, particularly in cells with large Ca^{2+} channel activity.

Sequential recording experiments also reveal that PC12 cell cultures were heterogeneous in their response to the spider toxin. This functional heterogeneity was characterized by the presence of a subset of cells apparently insensitive to ω-Aga-IVA during 6 min exposure. It is known that ω-Aga-IVA blockage of Ca^{2+} channel current in neurons is greatly time-dependent (Pearson et al., 1995; Randall and Tsien, 1995), thus differences in sensitivity to the toxin among PC12 cells could account for the lack of response observed in some cases: less susceptible cells could not be inhibited after short periods of toxin exposure. If this were the



B

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β1 PCRSEQ      1- GGGAGCCCTACCTTGTTTCCGGGGACCAGCCGCTGGACCGGGCCACTGGG
rat β1b     1394- .............................................

β1 PCRSEQ     51- GAGCATGCCAGTGTGCACGAGTACCCCGGGGAGCTGGGCCAGCCCCCAGG
rat β1b     1444- .............................................

β1 PCRSEQ    101- CCTTTACCCCAGCAACCACCCACCTGGCCGGGCAGGCACCCTGCGGGCGC
rat β1b     1494- .........................................T.....

β1 PCRSEQ    151- TATCCCGCCAAGACACCTTTGATGCTGACACCCCCGGCAGCCGAAACTCT
rat β1b     1544- .................................G...........

β1 PCRSEQ    201- GCCTACACGGAGCCAGGAGACTCGTGTGTGGACATGGAGACAGACCCCTC
rat β1b     1594- .T..........................................

β1 PCRSEQ    251- AGAGGGCCCAGGGCCTGGAGACCCTGCAGGGGGAGGTACACCACCAGCTC
rat β1b     1644- .............................................

β1 PCRSEQ    301- GGCAGGGCTCCTGGGAAGAGGAGGAAGATTATGAGGAGGAGATGACCGAC
rat β1b     1694- .............................................

β1 PCRSEQ    351- AACAGGAACCGGGGCCGGAATAAGGCCCGCTACTGTGCGGAGGGTGGTGG
rat β1b     1744- .............................................

β1 PCRSEQ    401- GCCGGTTCTGGGGCGCAATAAGAATGAGCTGGAGG
rat β1b     1794- ...................................
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Figure 4. Expression of mRNA of three Ca^{2+} channel β subunits in PC12 cells. *A*, RT-PCR from PC12 cell total RNA with β_1, β_2, and β_3 primers. *Left panel*, PCR products obtained from PC12 cDNA, control β_1, β_2, β_3, and β_4 cDNA with β_1 primers. *Middle panel*, PCR products amplified with β_2 primers from the same DNA samples as left panel. *Right panel*, PCR products amplified with β_3 primers from the same DNA samples as left panel. *B*, Alignment of β_1 sequence of PC12 RT-PCR product with rat β_{1b} sequence. The different nucleotides are in *italics*, and *dots* represent identical nucleotides.

case, long periods of time would be necessary to recruit the entire fraction of ω-Aga-IVA-sensitive cells; however, as illustrated in Figure 2*A*, blockage in PC12 cells is gradual, and Ca^{2+} channel activity runs down during continuous whole-cell recording, hampering the analysis of slow-developing effects of the toxin in individual cells. A second possibility could be that PC12 cultures were indeed composed of ω-Aga-IVA-sensitive and -insensitive cell subpopulations. The presence of subpopulations with differential expression of distinct Ca^{2+} channel types has been documented previously in neurons (Christenson et al., 1993) and endocrine cells (Felix et al., 1993) in culture. Likewise, a combination of both possibilities cannot be ruled out.

Taken together, these data indicate that ω-Aga-IVA partially inhibits a high-threshold Ca^{2+} current in undifferentiated PC12 cells and strongly suggest that a significant proportion of the current in these cells can be attributed to the activity of P/Q-type Ca^{2+} channels in their plasma membrane. Interestingly, when analyzed by subtraction (*lower trace* in Fig. 2*B*), the waveform of the current blocked by ω-Aga-IVA showed attributes described previously for the current induced by α_{1A} Ca^{2+} channel subunit expression in *Xenopus* oocytes (Sather et al., 1993; Stea et al., 1994; De Waard and Campbell, 1995), and it is comparable in relative magnitude and the degree of decay to the Q-type Ca^{2+} channel current in cerebellar neurons (Randall and Tsien, 1995). Although we have no information so far regarding the possible functional significance of the ω-Aga-IVA-sensitive current component in PC12 cells, it may be associated with neurotransmitter release as has been documented in chromaffin cells (Artalejo et al., 1994).

The presence of functional ω-Aga-IVA-sensitive Ca^{2+} current is in agreement with the expression of the α_{1A} subunit mRNA in undifferentiated PC12 cells. In addition, mRNA expression of α_{1B} and α_{1C} (isoform rbc-I) is consistent with the presence of N-type and L-type Ca^{2+} currents in PC12 cells, and the detection of specific receptors for ^{125}I-ω-CgTX (N-type) and [^{3}H]PN200-110 (L-type) in the PC12 membranes (data not shown). Therefore, the

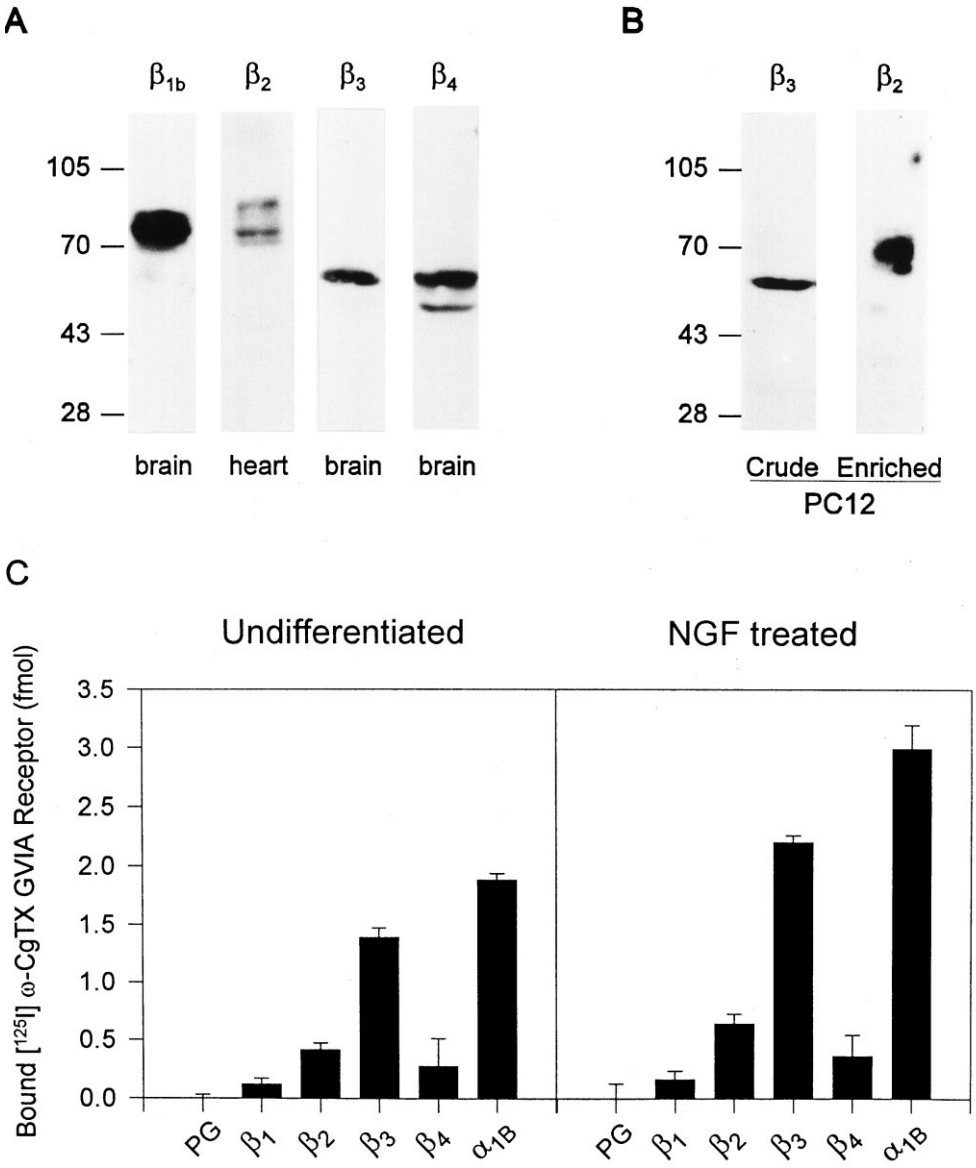

Figure 5. β_3 subunit is dominantly expressed and is the major form of β subunit of the N-type Ca^{2+} channels in PC12 cells. *A*, Four β subunit-specific antibodies recognize native β subunits from rat tissues. Shown are ECL immunoblots of crude rat tissue membranes stained by four affinity-purified β subunit-specific antibodies. Crude rat brain membranes were probed with polyclonal antibodies to β_1, β_3, and β_4 subunits, and rat cardiac microsome membranes were probed with polyclonal antibodies to β_2 subunit. *B*, Expression of β_3 and β_2 subunit protein in PC12 cells. Shown are an ECL immunoblot of crude PC12 membranes stained with affinity-purified β_3 subunit antibody and an ECL immunoblot of the eluate from a β_2 antibody affinity column stained with β_2 antibody. *C*, Association of different β subunits with the α_{1B} subunit of the PC12 N-type Ca^{2+} channel. Shown is the immunoprecipitation of [^{125}I]ω-CgTX GVIA receptors by protein G (*PG*), four β subunit antibodies, and polyclonal antibodies against α_{1B} subunit from undifferentiated and differentiated PC12 cells. The error bar represents the SE of three replicates.

expression of these different α_1 subunits provides the molecular basis for the heterogeneity of Ca^{2+} currents in PC12 cells. Furthermore, our data demonstrate that not only several α_1 subunits (α_{1A}, α_{1B}, and α_{1C}) but also multiple β subunits are expressed in the undifferentiated PC12 cells. Using a sensitive RT-PCR assay, mRNA expression of three β subunits (β_1, β_2, and β_3) was detected from PC12 cell. In contrast, control experiments from RNA without reverse transcription failed to amplify any signal, indicating that RT-PCR products were amplified from PC12 cDNA. The inability to detect β_1 in immunoblot analysis is probably attributable to technical difficulty because of a low expression level of Ca^{2+} channel subunits. The novel finding that three α_1 and three β subunits are expressed in PC12 cells suggests that multiple α_1 and β subunits are likely to be expressed in other neuronal cell types, because several different types of Ca^{2+} currents can be recorded from many neurons such as sympathetic neurons and cerebellum granule cells (Mintz and Bean, 1993; Pearson et al., 1995; Randall and Tsien, 1995). Considering the relative uniformity of PC12 cells, it is possible that a single PC12 cell may express multiple α_1 and β subunits, which raises the question how these α_1 and β subunits are assembled in a single cell.

Protein level of the Ca^{2+} channel subunits in PC12 cells is very low for immunoblot analysis. However, β_3 and an $\alpha_2\delta$ subunit (data not shown) can be detected on ECL immunoblot. There did not appear to be a significant change of expression level per milligram of total protein for β_3 or the $\alpha_2\delta$ subunit after NGF treatment. This may be attributable to the experimental error associated with ECL immunoblot analysis, which makes it difficult to detect less than a twofold change in protein level. On the other hand, immunoprecipitation experiments showed ~59% increase of the α_{1B} subunit after NGF treatment. Similarly, the level of the β subunits associated with the N-type channel increased in approximately the same proportion after NGF induction.

In our study, NGF treatment did not significantly alter the β subunit composition of the N-type Ca^{2+} channels despite an increase in total number of N-type channels. β_3 subunit remains the major β subunit of the N-type Ca^{2+} channel after NGF

treatment. This indicates that NGF regulates the expression of different β subunits in a similar manner. Furthermore, this suggests that NGF upregulates the expression of functional N-type channels without changing the properties of the channels.

Our results indicate that either β_3 or β_2 subunits are associated with the α_{1B} subunits of the N-type Ca^{2+} channel in PC12 cells, which is in agreement with the β subunit heterogeneity of N-type Ca^{2+} channels observed in rabbit brain (Scott et al., 1996). Although we detected the same order of association ($\beta_3 > \beta_4 > \beta_{1b}$) for different β subunits with the α_{1B} subunit in rat brain tissue as in rabbit brain (data not shown), the order of association for β subunits in PC12 cells ($\beta_3 > \beta_2$) is clearly different from that of mammalian brain. Whereas numerous neuronal cell types are present in brain, PC12 cells are rather homogeneous, although individual cells may differ to some extent. It is therefore important to demonstrate that in a single cell type, the α_{1B} subunit is associated with either of the two β subunits. In addition, this is the first evidence that β_2 subunit can associate with the N-type channel. Noticeably, the association of β_3 subunit with the α_{1B} subunit correlates with the dominant expression of this subunit in PC12 cells suggesting a role for the expression level of β subunits in determining the β subunit association with the calcium channels.

The diversity of Ca^{2+} channel currents in PC12 cells arise from the expression of at least three α_1 subunits and three β subunits. Whereas α_1 subunits determine the major properties of the current type (N, L, P/Q), different β subunits may further modify the properties of the Ca^{2+} channels. In support of a previous finding that three of brain β subunits are associated with the N-type channel (Scott et al., 1996), at least two populations of N-type channels (α_{1B} β_3 and α_{1B} β_2) are present in a single PC12 cell line that may differ in their function and/or localization. However, NGF does not appear to modify the fraction of each population over the 3 d period we examined. Therefore, the properties of the N-type channels resulting from the β subunit association remain unchanged before and after NGF induced differentiation. In the future, PC12 cells can serve as a neuronal cell model for additional investigation of the regulation and function of N-, L-, and P/Q-type Ca^{2+} channels during development.

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