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# The Molecular Identity of Ca Channel $\alpha_1$-Subunits Expressed in Rat Sympathetic Neurons

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## Abstract

Much of our understanding of the mechanisms of the gating, modulation, and function of neuronal Ca channels has its origins in investigations of sympathetic neurons. In this article, we use molecular analyses to identify the three Ca channel $\alpha_1$-subunits that are the likely counterparts to the pharmacologically defined: ω-Conotoxin GVIA-sensitive N-type; dihydropyridine-sensitive L-type, and ω-Conotoxin GVIA-insensitive, dihydropyridine-insensitive Ca channel currents observed in sympathetic neurons. With a combination of degenerate and exact primers, small regions of Ca channel $\alpha_1$-subunit sequences were amplified by the polymerase chain reaction (PCR). Although all five Ca channel $\alpha_1$-subunit genes were expressed in rat sympathetic ganglia, $\alpha_{1B}$-, $\alpha_{1D}$-, and $\alpha_{1E}$-derived cDNAs were the dominant species. No novel Ca channel $\alpha_1$-sequences were identified in the regions selected for amplification, and we conclude that $\alpha_{1B}$, $\alpha_{1D}$, and $\alpha_{1E}$ likely encode, respectively, N-type, L-type, and non-N/non-L-type channel currents of rat sympathetic neurons. In addition, we show that Ca channel $\beta_2$-, $\beta_3$-, and $\beta_4$-subunit sequences are strongly represented in sympathetic ganglia. The results of this study also suggest that $\alpha_{1D}$, and not $\alpha_{1C}$, regulates Ca influx through dihydropyridine-sensitive Ca channel currents.

**Index Entries:** Calcium channels; sympathetic neurons; polymerase chain reaction; calcium channel $\alpha_1$-subunits; ω-conotoxin GVIA; dihydropyridine; nickel.

## Introduction

Voltage-gated calcium channels are the gateway for calcium ions entering the neuron in response to membrane depolarization. As such, they control an impressive array of critical neuronal functions, including neurotransmitter release, neurite outgrowth and gene expression (Miller, 1987; Murphey et al., 1991; Komuro and Rakic, 1992; Artalejo et al., 1994; Dunlap et al., 1995). Consis-

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tent with their central role in regulating many different cellular functions, voltage-gated calcium channels are members of a large protein family, the main subunit of which ($\alpha_1$; Tanabe et al., 1987) is encoded by at least six distinct genes, most commonly referred to as $\alpha_{1A-E}$ and $\alpha_{1S}$ (Snutch and Reiner, 1992; Birnbaumer et al., 1994; Dunlap et al., 1995). Five of these genes, $\alpha_{1A-E}$, are expressed in the mammalian brain and heterologous expression in frog oocytes (Mori et al., 1991; Tomlinson et al., 1993; Stea et al., 1993; Soong et al., 1993), human embryonic kidney cells (Williams et al., 1992a, b), and dysgenic mouse muscle (Fujita et al., 1993) have confirmed that these genes encode neuronal-like voltage-gated Ca channels. At least three other subunits, β, $\alpha_2$, δ, are thought to associate with the $\alpha_1$-subunit (Ahlijanian et al., 1990; Witcher et al., 1993) with most attention currently focused on the cytoplasmically located β-subunit which appears to increase the coupling ratio between the voltage-sensing and channel-gating mechanisms of the $\alpha_1$-subunit (Neely et al., 1993). The binding domains that specify the interaction of the β-subunit with $\alpha_1$ have been localized to a series of conserved residues within each protein family (DeWaard et al., 1994; Pragnell et al., 1994), but interestingly, there is considerable flexibility in this interaction exemplified by the observation that different β-subunits ($\beta_{1-4}$; Pragnell et al., 1991; Perez-Reyes et al., 1992; Castellano et al., 1993a, b) can interact with and regulate a single class of $\alpha_1$-subunit in heterologous expression systems (*see* Isom et al., 1994 for review) as well as in the native cell (Scott et al., 1996).

Much effort has now shifted to an assessment of the functional importance of the various Ca channel $\alpha_1$- and β-subunit clones isolated from the mammalian nervous system. In some cases, where selective toxins are available, the initial stage of characterization has been relatively straightforward. For example, with the N-type Ca channel, the use of ω-CgTx GVIA has shown that $\alpha_{1B}$ encodes an ω-CgTx-GVIA-sensitive ICa channel current (Williams et al., 1992a; Fujita et al., 1993; Stea et al., 1993). Likewise, the use of dihydropyridines (DHPs) confirmed that L-type Ca channels of neurons are encoded by two different genes, $\alpha_{1C}$ and $\alpha_{1D}$ (Williams et al., 1992b; Tomlinson et al., 1993). However, identifying the molecular counterparts to P-type and T-type Ca channels and, on the other hand, assigning functional partners to $\alpha_{1A}$- and $\alpha_{1E}$-subunits, all of which are expressed in the mammalian brain, has not been so simple (Sather et al., 1993; Soong et al., 1993; Zhang et al., 1993; Dunlap et al., 1995).

In this article, we characterize the molecular components underlying Ca channel currents in the superior cervical ganglia of the rat. Sympathetic neurons represent one of the best-characterized groups of cells in the mammalian nervous system, and much of our knowledge of Ca channel gating, modulation, and function has its origins in electrophysiological studies of these cells (McCleskey et al., 1987; Hirning et al., 1988; Plummer et al., 1989; Ikeda, 1991; Mathie et al., 1992; Boland et al., 1994).

## Experimental Procedures

### *Whole-Cell Recording*

Recordings were obtained from sympathetic neurons, isolated from superior cervical ganglia of 4-d-old rats, either immediately following isolation or 2–3 d in culture. Tissue-isolation and tissue-culture procedures were performed as described in Hawrot and Patterson (1979). Macroscopic Ca channel currents were recorded with the whole-cell patch-clamp technique (Hamill et al., 1981). Electrodes of 1.5–2 MΩ were filled with 100 m*M* CsCl; 10 m*M* EGTA; 40 m*M* HEPES; 2 m*M* ATP, 5 m*M* $MgCl_2$, pH adjusted to 7.4 with CsOH. The bathing solution contained 135 m*M* TEACl, 2 m*M* $BaCl_2$, and 5 m*M* HEPES, pH adjusted to 7.4 with TEAOH. Voltage pulses were generated and currents digitized and sampled on-line using Pclamp V6 (Axon Instruments). Leak and capacitative currents were subtracted using a P/4 protocol. Whole-cell capacitance and series resistances were compensated >85% with the use of the Axopatch 200 (Axon Instruments) and continually monitored throughout the recording period.

### *Isolation of RNA from Rat Superior Cervical Ganglia (SCG)*

Superior cervical ganglia (SCG) and brains were rapidly removed from a total of 120, 4 d-old rats (Sprague Dawley), immediately frozen in liquid $N_2$, and used within 2 d. mRNA was purified from the

tissue using the Quick Prep mRNA Purification Kit (Pharmacia). Briefly, frozen tissue was placed in an extraction buffer containing guanidium thiocyanate and *N*-lauroyl sarcosine, and immediately homogenized (polytron PT1200, Brinkman). mRNA was purified by passage twice through an oligo dT cellulose spin column. Fourteen micrograms of mRNA were obtained from approx 240 ganglia, and 13 μg mRNA from 180 mg of brain tissue; 2.5 μg of mRNA isolated from each tissue were run on a 0.8% agarose gel containing 2.2*M* formaldehyde, which indicated the presence of significant amounts of RNA of over 10 kb.

## Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

First-strand cDNA synthesis was oligo dT- and random-primed and transcribed using the Invitrogen cDNA Cycle Kit. The reverse transcription was performed in a 20-μL mix containing 800 ng of SCG mRNA, 1X RT buffer, 1.0 μg of random primer, 0.2 μg of oligo dT primer, 5 m*M* each deoxynucleotide triphosphate, 4 m*M* sodium pyrophosphate, 5 U of RNase inhibitor, and 2.5 U of reverse transcriptase. To increase the yield of low abundance message, cDNA was synthesized over a 120-min incubation at 42°C and an additional 2.5 U of reverse transcriptase was added at the 60-min time point. PCR amplification proceeded in a 50-μL reaction mix containing 1–4 μL of cDNA, 1.25 U of *Taq* DNA polymerase (Promega), 50 m*M* KCl, 10 m*M* Tris-HCl, pH 9.0, 0.1% Triton X-100, 1.5 m*M* $MgCl_2$, 200 m*M* of each deoxynucleotide triphosphate, and 0.2 μ*M* of the appropriate upstream and downstream primers,. Following a 2-min incubation at 95°C, cDNA amplification was achieved by cycling 30 times at 95°C for 1 min, 52°C for 1.5 min, and 74°C for 3 min. The reaction was terminated by a 7-min incubation at 72°C. Amplified cDNA products were separated on a 1.5% agarose gel. For cDNA amplification using degenerate primers, the reaction mix was as above, except 4 μL of the cDNA and 1 μg of the upstream and downstream degenerate primer pool were added. The degenerate amplification cycle was initiated by incubation at 95°C for 5 min, 52°C for 2 min, and 74°C for 1 min, followed by 38 cycles of 95°C for 1 min, 52°C for 2 min, 74°C for 1 min, and terminated by 95°C for 1 min, 52°C for 2 min, and 74°C for 10 min. Negative controls were routinely performed to ensure that our reagents were not contaminated with Ca channel cDNA or mRNA. This was achieved by adding enzyme, nucleotides and primers in the absence of template.

### Primer Sequences

We synthesized several different sets of degenerate primers that targeted several homologous regions of calcium channel $\alpha_1$-subunit cDNAs ($\alpha_1$). Of the pairs of primer pools tested, $\alpha_1$126 was used with the most success and amplified a region that could, by restriction analysis, distinguish all five $\alpha_1$–subunit cDNAs. Primer sequences were as follows: $\alpha_{1A-E}$ degenerate primers: $\alpha_1$u126: 5'-AT{T/C/A}GG{T/C/A/G}ATGCA{A/G}GT{T/C/A/G}TT{T/C}GG-3' (192 different primers); $\alpha_1$d126: 5'-CCA{T/C/A/G}GC{T/C}TC{T/C/A/G}CC{T/C/A/G}GT{T/C/A/G}GC-3' (512 different primers). The rat brain $\alpha_1$- and β-specific primers used in this study and the predicted size of cDNA products were as follows: rb$\alpha_{1A-E}$ specific primers: $\alpha_{1A}$u5041: 5'-ATGAGTTCCAAATCACGGAGC-3'; $\alpha_{1A}$d5520: 5'-GTGGAGTTGAAGTGAACGGTG-3' (504-bp product; Starr et al., 1991); $\alpha_{1B}$u5015: 5'-CACATGCCAACGCCAGCGAATG-3', $\alpha_{1B}$d5623: 5'GACAGGCCTCCAGGAGCTTGGTG-3' (633-bp product; Dubel et al., 1992); $\alpha_{1C}$u4153: 5'-ATTGCCCTGAATGACACCACAG-3', $\alpha_{1C}$d4929: 5'-GACAGTGCATTCCTCTGCGAG-3' (799-bp product; Snutch et al., 1991); $\alpha_{1D}$u4264: 5'-CCGGACTCAGATTACAACCCAG-3', $\alpha_{1D}$d4895: 5'-TAGGGCGATCGTGGTGTTCTTCG-3' (656-bp product; Hui et al., 1991); $\alpha_{1E}$u4918: 5'-CAGAACGAGAGTGAGCGCTGTG-3', $\alpha_{1E}$d5684: 5'-CCAACTGGAATATTTCTTGAGG-3' (789-bp product; Soong et al., 1993). rb$\beta_{1b}$–rb$\beta_4$ specific primers: $\beta_{1b}$u1384: 5'-CATGCCAGTGTGCACGAGTACC-3', $\beta_{1b}$d1746: 5'-GCCCTCCAGC TCATTCTTATTG-3' (384-bp product; Pragnell et al., 1991); $\beta_2$u1336: 5'-GGTGATCAGAGGACTGATCGCTC-3', $\beta_2$d1656: 5'-GTCTCGACCCATGTCCCTAGTG-3' (342-bp product; Perez-Reyes et al., 1992); $\beta_3$u1119: 5'-CAGTGCCATCCCTGGACTTCAG-3', $\beta_3$d1416: 5'-CCTTAGGCCAAGGCCGGTTACG-3' (319-bp product; Castellano et al., 1993a); $\beta_4$u1245: 5'-GAACGTGGGCTCCACAGCTCTC-3', $\beta_4$d1529: 5'-TGTCGGGAGTCATGGCTGCATC-3' (306-bp product; Castellano et al., 1993b).

### Subcloning and Sequencing PCR Products

The degenerate PCR-derived cDNAs were directly ligated into pT7Blue® T-vector (Novagen) and the plasmids transformed into NovaBlue competent cells grown on LB/tet/amp plates. A blue/white screening identified 30 positive recombinants (white), 24 of which were the appropriate size (126 or 150 bp) for potential Ca channel $\alpha_1$ cDNAs and thus selected for further analysis. Restriction analysis was used as a first-pass screen to identify $\alpha_{1B}$-derived cDNA. Clones that yielded unidentifiable digest patterns were directly sequenced using Sequenase V2.0 (USB).

## Results

### Sympathetic Neurons Express Three Pharmacologically Distinct Ca Channel Currents

The results shown in Fig. 1 indicate that sympathetic neurons from 4–5-d-old rats, used in the present study, express three pharmacologically distinct Ca channel currents. It is well known that sympathetic neurons express a robust whole-cell Ca current that is dominated by an ω-CgTx-GVIA-sensitive, N-type current. The N-type current is evoked by strong depolarizations from relatively negative holding potentials (Fig. 1, *see also* McCleskey et al., 1987; Plummer et al., 1989; Ikeda, 1991; Boland et al., 1994) and, as shown in Fig. 1, is irreversibly inhibited by 200 n*M* ω-CgTx GVIA. In addition to the N current, it has also been established that rat sympathetic neurons express two ω-CgTx-insensitive Ca currents (Plummer et al., 1989; Boland et al., 1994). One, L-type, is sensitive to DHPs, and the other, which is resistant both to DHPs and to ω-CgTx GVIA, is reversibly inhibited by 50–200 μ*M* $Ni^{2+}$ (*see* Fig. 1; *see also* Elmslie et al., 1994). Figure 1 highlights the basic properties of these two currents. Nimodipine (1–2 μ*M*) reversibly inhibits 10–20% of the ω-CgTx GVIA-insensitive Ca channel current, whereas the remaining "insensitive" current was inhibited by 50 μ*M* $Ni^{2+}$. Thus, based on pharmacological criteria, rat sympathetic neurons express three distinct Ca channel currents: N-type, L-type, and an as-yet uncharacterized component.

### RT-PCR Screening of Ca Channel $\alpha_1$-Subunits

As a first step toward determining the molecular counterparts of these three components of the Ca channel current, we screened, by RT-PCR, mRNA obtained from rat superior cervical ganglia, using five sets of primers targeting the unique C-terminal domains of each of the $\alpha_{1A-E}$ subunits. We focused our studies on the $\alpha_1$-subunit of the Ca channel complex, since it comprises the ion channel-forming subunit. In addition, the $\alpha_1$-subunit appears to be responsible for specifying the kinetic and pharmacological determinants commonly used to distinguish among members of the voltage-gated Ca channel family (*see* Tsien et al., 1991). Figure 2 shows that after 30 rounds of DNA amplification, sequences corresponding to all five transcripts encoding $\alpha_{1A-E}$ were detected in mRNA from both SCG and brain of newborn rats. In SCG, in contrast to brain, it is the $\alpha_{1B}$-derived PCR product that is clearly most prominent. The relatively strong signal obtained using the $\alpha_{1B}$ primers and SCG mRNA-derived cDNA as template is expected given that $\alpha_{1B}$ gene encodes the N-type Ca channel (Dubel et al., 1992; Williams et al., 1992a), which is expressed at relatively high levels in sympathetic neurons (McCleskey et al., 1987; Boland et al, 1994). The high efficiency of SCG cDNA amplification using the $\alpha_{1D}$ primers, compared to $\alpha_{1C}$, is also noteworthy, since both $\alpha_{1C}$ and $\alpha_{1D}$ cDNAs have been shown to encode a DHP-sensitive L-type Ca channel (*see* Tsien et al., 1991). Several lines of evidence suggest that $\alpha_{1D}$ is expressed in greater abundance than $\alpha_{1C}$ in the ganglia, specifically:

1. The same result, $\alpha_{1B} > \alpha_{1D} \approx \alpha_{1E} > \alpha_{1A} > \alpha_{1C}$ (Fig. 2A) was obtained using the same primers, but with mRNA prepared from a different group of animals.
2. The signal owing to $\alpha_{1D}$-cDNA was still much greater than the $\alpha_{1C}$-cDNA signal when two different primer pairs were used.
3. Under a variety of different PCR conditions, the signal owing to $\alpha_{1B}$-derived cDNA was always the greatest, and $\alpha_{1D}$ primers always gave a stronger signal than $\alpha_{1C}$. This was true even after diluting first-strand cDNA by 2-, 10-, and 100-fold (Fig. 3), and analyzing samples after 15, 20, 25, and 30 amplification cycles with these three template concen-

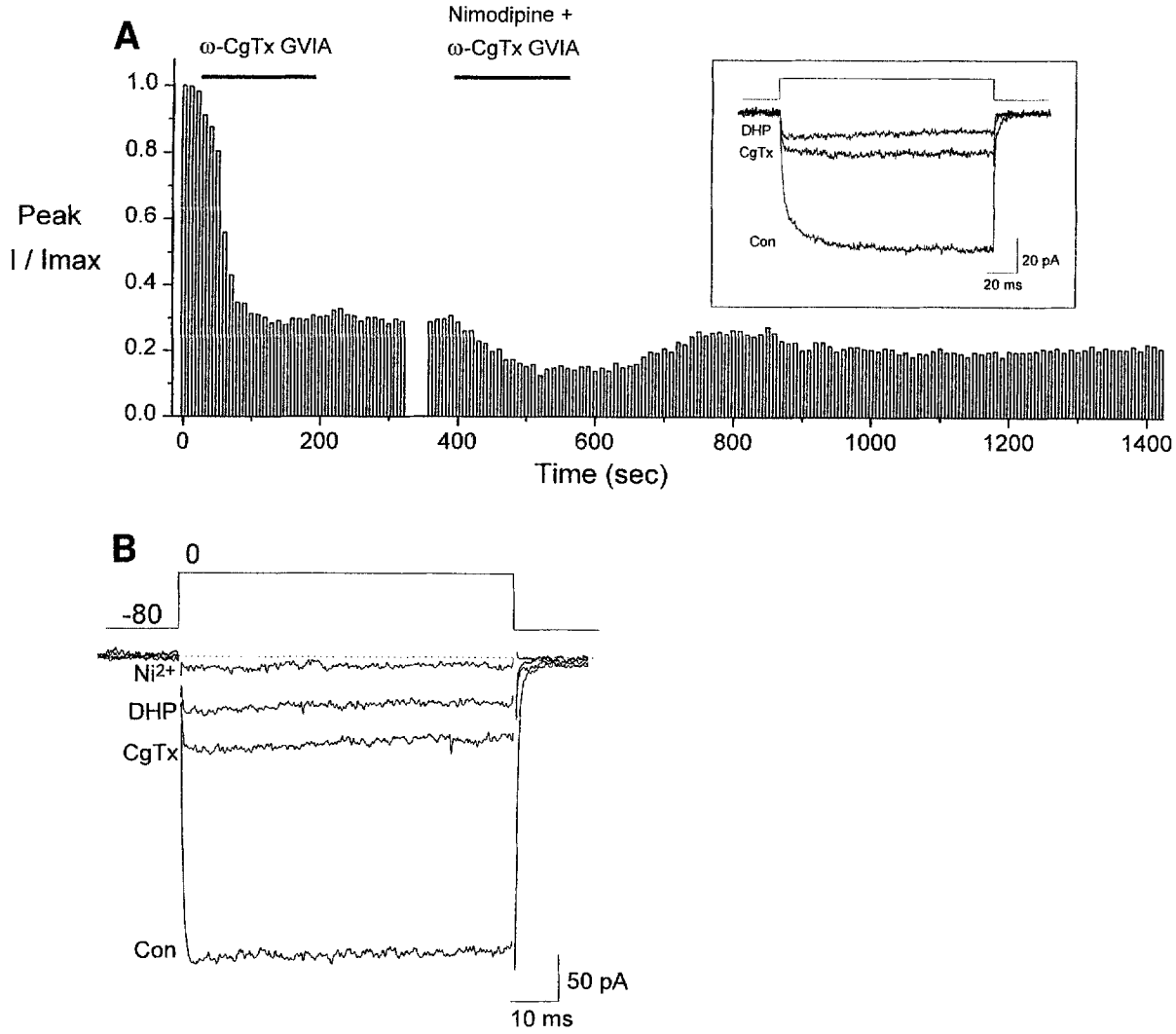


Fig. 1. Rat sympathetic neurons express three pharmacologically distinct Ca channel currents. Whole-cell Ca channel currents recorded from two sympathetic neurons isolated from the superior cervical ganglia of newborn rats. Currents were recorded using 2 m*M* $Ba^{2+}$ as the charge carrier. **(A)** Time-course of peak current amplitude. Currents were evoked by voltage steps to –10 mV from a holding potential of –80 mV applied every 10 s. Bars indicate time of exposure to 200 n*M* ω-CgTx GVIA and 200 n*M* ω-CgTx GVIA plus 1 μ*M* nimodipine. Approximately 70% of the current was rapidly and irreversibly inhibited by ω-CgTx GVIA. There was no reversibility of inhibition by ω-CgTx GVIA after 30 min of washing in toxin-free solution (first 15 min shown here). Inset shows individual currents recorded from the same sympathetic neuron. **(B)** Individual currents recorded from a different sympathetic neuron evoked by step depolarizations to 0 mV from a holding potential of –80 mV; 200 n*M* ω-CgTx GVIA, 2 μ*M* nimodipine, and 50 μ*M* $Ni^{2+}$ were applied successively, in an additive manner, once the current amplitude was steady. Leak and capacitative currents were subtracted on line using a P/4 protocol and currents were filtered at 1 kHz (–3 dB).

trations. In fact, the PCR product owing to $\alpha_{1C}$ cDNA was detectable only following PCR amplification using the highest concentrations of template (1:1 and 1:2 dilution) and then only after 30 cycles of amplification (Figs. 2A and 3).

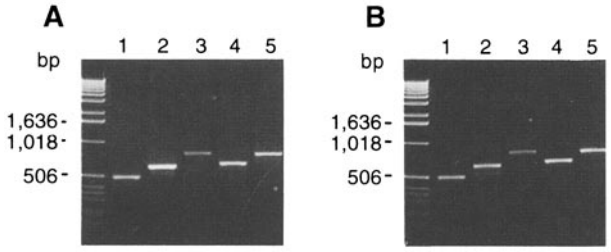


Fig. 2. Transcripts for all five calcium channel $\alpha_1$-subunits can be detected by RT-PCR amplification from mRNA isolated from superior cervical ganglia and brains of newborn rats. Lanes 1–5 are the PCR-derived 500–800 bp cDNA products obtained using five sets of rat brain $\alpha_{1A-E}$ specific primers, respectively, using 40 ng of SCG **(A)** and brain **(B)** mRNA as the starting template. In both SCG and brain, a single band, close to the predicted cDNA size, dominated in each of the five lanes. Lane 1, primers $\alpha_{1A}$u5041 and $\alpha_{1A}$d5520 yielded a single band at ~504 bp; lane 2, primers $\alpha_{1B}$u5015 and $\alpha_{1B}$d5623, a major product at ~633 bp; lane 3, primers $\alpha_{1C}$u4153 and $\alpha_{1C}$d4929, a single band at ~799 bp; lane 4, primers $\alpha_{1D}$u4264 and $\alpha_{1D}$d4895, a single product at ~656 bp, lane 5, primers $\alpha_{1E}$u4918 and $\alpha_{1E}$d5684, a single product at ~789 bp. Double-stranded cDNA markers are shown in the first lane of each gel. Negative controls were performed in the absence of template, and no product was amplified.

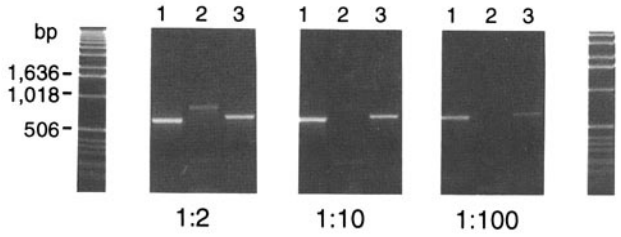


Fig. 3. Effect of decreasing template concentration on the PCR amplification efficiency using rat brain $\alpha_{1B}$-, $\alpha_{1C}$-, and $\alpha_{1D}$-specific primers. SCG-derived cDNA was diluted by 1:2, 1:10 and 1:100 (indicated at the lower edge of each gel) and used as the PCR template. Three sets of unique primers targeting rat brain $\alpha_{1B}$ (lane 1), $\alpha_{1C}$ (lane 2), and $\alpha_{1D}$ (lane 3) were used to PCR amplify from each of the three cDNA concentrations. The PCR-derived cDNA products obtained after 30 amplification cycles are shown separated on 1% agarose gels. Two cDNA markers are shown bracketing the three gels and DNA sizes are indicated alongside. $\alpha_{1C}$-derived cDNA was only visible at the highest cDNA concentration (1:2); in contrast, $\alpha_{1B}$- and $\alpha_{1D}$-derived cDNAs were observed following a 1000-fold dilution. The strongest signal was obtained with the $\alpha_{1B}$-specific primers.

## *Degenerate RT-PCR Identifies the Most Abundantly Expressed Ca Channel $\alpha_1$-Subunits*

PCR amplification using exact primers directed to rat brain $\alpha_{1A-E}$ provided a uniquely efficient method for detecting even minute quantities of template. The results are consistent with $\alpha_{1B}$ being the most abundant and $\alpha_{1C}$ the least abundant of the Ca channel $\alpha_1$-subunit transcripts in sympathetic ganglia. To test this hypothesis further, we used degenerate PCR primers that, to a first approximation, will preferentially amplify the most abundant message because of competition for nucleotides and DNA polymerase, to amplify a small region of 126–150 bp within the hypothesized pore-forming region IVS5–IVS6 of the Ca channel $\alpha_1$-subunit. As shown in Fig. 4, a prominent cDNA band of about 126 bp, the predicted size for $\alpha_{1B-E}$ (*see* Experimental Procedures), was identified following PCR amplification of SCG and brain cDNAs, as well from the rat brain-derived rb$\alpha_{1B}$ clone (rbB-1; Dubel et al., 1992). There was no visible band in the region of 150 bp, the size predicted for $\alpha_{1A}$-derived cDNAs, using the $\alpha_1$-degenerate primers (Fig. 4). In order to establish the $\alpha_1$-subtype composition of the SCG cDNAs, we subcloned the PCR product pool into pT7Blue, performed a second round of confirmatory PCR on individual clones, and then positively identified each by a combination of restriction analysis and DNA sequencing. From a total of 30 clones, 24 were found to be of the appropriate size for potential Ca channel $\alpha_1$ cDNAs, and of these, 19 were confirmed to belong to this gene family. Positive identification by DNA sequencing and restriction digest analysis revealed that 74% (14/19) belonged to the $\alpha_{1B}$ class, 21% (4/19) to $\alpha_{1D}$, and 5% to $\alpha_{1E}$ (1/19). No clones corresponding to the $\alpha_{1C}$ or $\alpha_{1A}$ family were identified and no new variants of $\alpha_1$ were observed in this region of the coding sequence. These results are remarkably consistent with the expected composition of the mRNA pool based on the

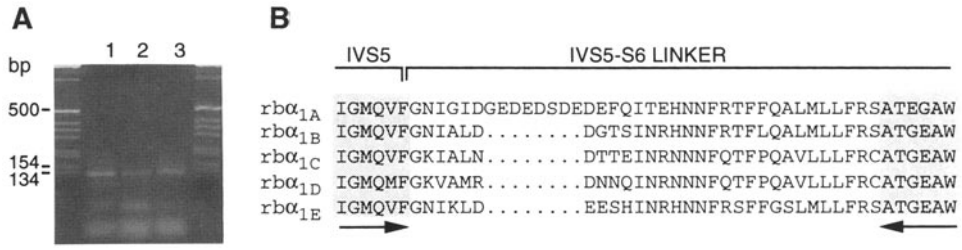


Fig. 4. Degenerate RT-PCR selectively amplifies cDNA derived from $\alpha_{1B}$-, $\alpha_{1D}$-, and $\alpha_{1E}$-subunits from rat SCG mRNA. **(A)** RT-PCR amplification within the putative IVS5-IVS6 region of the Ca channel $\alpha_1$-subunit. Forty nanograms of rat SCG mRNA (lane 1) and 40 ng of brain mRNA (lane 2) were used as initial templates for first-strand DNA synthesis and full-length $rb\alpha_{B-1}$ cDNA (lane 3) as control. Two large pools of degenerate $\alpha_1$ primers were used in the amplification reaction, and products shown were size-separated on a 1% agarose gel. A band is clearly visible at ~126 bp in each reaction. The two lower mol-wt diffuse bands are consistent with the presence of a large pool of unused primers and primer dimers. Outside lanes contain double-stranded cDNA size markers. **(B)** Published amino acid sequences of rat brain $\alpha_{1A-E}$ subunits within the putative IVS5-IVS6 pore-forming region (Starr et al., 1991; Snutch et al., 1991; Hui et al., 1991; Dubel et al., 1992; Soong et al., 1993). The regions selected for PCR amplification are shown, and the location of the primers delineated by the arrows. The predicted sizes of PCR-derived cDNAs are 126 bp for $\alpha_{1B-E}$ and 150 bp for $\alpha_{1A}$.

pharmacological analysis of whole-cell Ca channel currents (Fig. 1). Characterizing individual PCR-derived cDNAs, in order to determine the relative abundance of particular transcripts in the mRNA pool, has proven useful in studies of other neuronal ion channels (e.g., *see* Ruano et al., 1995 for quantification of distinct kainate receptor mRNAs in single hippocampal neurons).

### *Identification of Ca Channel β-Subunits in Rat SCG*

The $\alpha_1$-subunit of the Ca channel apparently requires several other accessory proteins for normal gating and function. One important component of the macromolecular complex comprising the functional Ca channel is the β-subunit, which has been shown to affect the gating (*see* Isom et al., 1994) and modulation (e.g., Stea et al., 1995) of the Ca channel. We therefore set out to determine which, if any, of the four previously characterized β-subunit genes expressed in rat brain (Pragnell et al., 1991; Perez-Reyes et al., 1992; Castellano et al., 1993a,b) were also expressed in rat SCG. Four sets of unique PCR primers, targeting a variant stretch of amino acids within the rat brain $\beta_{1b}$–$\beta_4$ sequences (*see* Experimental Procedures), were used to screen mRNA isolated from SCG or brains of newborn rats. The results shown in Fig. 5 are consistent with all four β-subunits being expressed in SCG, although a relatively weak signal was obtained with the $\beta_{1b}$-specific primers. In contrast, the $\beta_{1b}$-primers were almost as effective as the other primer sets in amplifying Ca channel β-subunit cDNA from brain.

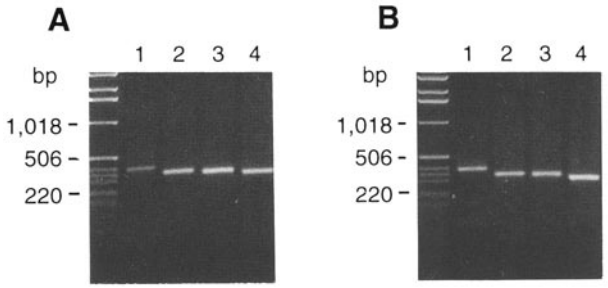


Fig. 5. Evidence for the presence of mRNA encoding all four calcium channel β-subunits in rat SCG and brain by RT-PCR. Forty nanograms of mRNA from SCG **(A)** and 40 ng of mRNA from brain **(B)** were used as templates for RT-PCR using four sets of unique primers targeting $\beta_{1-4}$ (lanes 2–5). Four major PCR-derived cDNA products were observed on each gel as indicated by the presence of single bands at each of the predicted sizes 385, 342, 319, and 306 bp for $\beta_{1-4}$, respectively. The cDNA sizing marker is shown in the first lane of each gel. PCR products were separated by electrophoresis in 1% agarose.

## Discussion

### Correlating PCR-Derived cDNAs with Whole Ca Channel Currents

Our analysis of mRNA derived from rat SCG suggests that three distinct $\alpha_1$-subunits are preferentially expressed in this tissue, the relative abundance of which, $\alpha_{1B}$ (74%), $\alpha_{1D}$ (21%), and $\alpha_1$E (5%), is consistent with electrophysiological analysis of whole-cell Ca channel currents recorded from rat sympathetic neurons (Fig. 1; Boland et al., 1994). Our data, together with that of others, suggest that 65–95% of the macroscopic Ca channel current evoked by strong depolarizations in sympathetic neurons is an ω-CgTx GVIA-sensitive, N-type current, that 10–20% is a DHP-sensitive, L-type current, and that 5–15% of Ca channel current remains in the presence of ω-CgTx GVIA and DHP antagonists (Hirning et al., 1988; Plummer et al., 1989; Ikeda, 1991; Boland et al., 1994; *see also* Elmslie et al., 1994). cDNAs corresponding to $\alpha_{1C}$ and $\alpha_{1A}$ were detected only after 30 cycles of PCR amplification using $\alpha_{1C}$- and $\alpha_{1A}$-specific primers. The relatively weak amplification of these cDNAs, together with their absence from PCR-derived cDNA pools using $\alpha_1$ degenerate primers, suggests that Ca channel $\alpha_{1A}$- and $\alpha_{1C}$-subunits are expressed in very low abundance in the ganglia of newborn rats. Consistent with the low expression of $\alpha_{1A}$, which encodes an ω-Aga IVA-sensitive Ca channel current (Mori et al., 1991), are electrophysiological studies in rat sympathetic neurons that report complete insensitivity of whole-cell Ca channel currents to high concentrations of the $\alpha_{1A}$-specific inhibitor, ω-Aga IVA (Mintz et al., 1992; Mintz and Bean, 1993; Zhu and Ikeda, 1993). Since $\alpha_{1C}$ and $\alpha_{1D}$ both encode a DHP-sensitive L-type Ca channel current, the low expression of $\alpha_{1C}$ mRNA in rat SCG could not have been predicted from pharmacological dissection of whole-cell Ca channel currents *(see below)*.

### Does $\alpha_{1E}$ Encode the ω-CgTx GVIA-Insensitive, DHP-Insensitive Ca Channel Current?

The presence of a DHP-resistant, ω-CgTx GVIA-resistant component of whole-cell Ca channel current has been noted by others in studies of both rat (Plummer et al., 1989; Boland et al., 1994) and frog (Elmslie et al., 1994) sympathetic neurons. It has been difficult to identify this component of the whole-cell Ca channel current unambiguously because of the lack of a specific toxin to inhibit its activity selectively. Nonetheless,

1. The absence of sensitivity to known toxins, which selectively inhibit both native and heterologously expressed N-type ($\alpha_{1B}$), L-type ($\alpha_{1C}$ and $\alpha_{1D}$), and P/Q-type ($\alpha_{1A}$);
2. The relatively high sensitivity of this component of the Ca current to inhibition by low concentrations of $Ni^{2+}$ (50–200 μM; Fig. 1 and *see* Elmslie et al., 1994);
3. The fact that $\alpha_{1E}$-derived cDNA was isolated from the degenerate PCR; and
4. The observations that heterologously expressed $\alpha_{1E}$Ca channel currents are inhibited by low concentrations of $Ni^{2+}$ (rbE-II, Soong et al, 1993; Doe-1, Ellinor et al., 1993).

taken together suggest that the ω-CgTx GVIA-resistant, DHP-resistant component of whole-cell Ca channel current expressed in sympathetic neurons is encoded by $\alpha_{1E}$.

### $\alpha_{1D}$ Encodes the DHP-Sensitive L-Type Ca Channel Current in Newborn Rat SCG

Our results suggest that the DHP-sensitive Ca channel current expressed in newborn rat sympathetic neurons is encoded by $\alpha_{1D}$, but not $\alpha_{1C}$. This result could not have been predicted from analysis of whole-cell Ca channel currents, since $\alpha_{1D}$ and $\alpha_{1C}$ both encode DHP-sensitive Ca channel currents that are, to a first approximation, pharmacologically and kinetically similar (Williams et al., 1992b; Tomlinson et al., 1993). Only the report from Williams et al. (1992b) that human $\alpha_{1D}$ currents expressed in frog oocytes are weakly and reversibly inhibited by high concentrations of ω-CgTx GVIA currently suggests a way of separating $\alpha_{1C}$- from $\alpha_{1D}$-type currents. However, Boland et al. (1994) failed to observe any reversibility of ω-CgTx GVIA block of Ca currents in rat sympathetic neurons using very high toxin concentrations up to 100 μM, consistent with our findings using lower concentrations of toxin (200 nM; Fig. 1). In the one report of reversibility of ω-CgTx GVIA inhibition of Ca channel currents in rat sympathetic neurons, an involvement of the L-type Ca

channel family was ruled out by showing that this component was insensitive to DHP (Plummer et al., 1989). Further studies will be necessary to establish how similar the properties of the heterologously expressed $\alpha_{1D}$ currents are to the native channel. Studies of L-type Ca channel currents in rat sympathetic neurons might provide a useful system to make this comparison, since presumably $\alpha_{1D}$ is exclusively expressed in these cells.

The predominant somatic localization of $\alpha_{1C}$- and $\alpha_{1D}$-subunits in central neurons (Hell et al., 1993) is consistent with a recent study showing that L channels in rat sympathetic neurons stimulate the expression of the $\alpha_7$-subunit of the neuronal nicotinic receptor (De Koninck and Cooper, 1995). Our results would suggest that the $\alpha_{1D}$-class of L-type channel mediates this response in sympathetic neurons.

It is quite possible that during development and at maturity, sympathetic neurons express a different constellation of Ca channel genes than those expressed in the neonate. Indeed, Tanaka and Koike (1995) have recently shown that expression of $\alpha_{1C}$ mRNA is upregulated in rat superior cervical ganglia within 2–5 d following exposure to nerve growth factor. Consistent with our results, however, Tanaka and Koike (1995) report low levels of expression of $\alpha_{1C}$ in the ganglia of newborn rats, although in their study they did not test for the presence of other $\alpha_1$-subunits.

### $\alpha_{1B}$ Encodes the Major Component of the N-Type Ca Channel Current

The N-type Ca channel has been shown to be the major route of entry Ca-regulating catecholamine release in sympathetic neurons (Hirning et al., 1988), and our results, which suggest that $\alpha_{1B}$ is the most abundant of the Ca channel $\alpha_1$-subunits expressed in sympathetic neurons, are consistent with this. Although PCR-derived $\alpha_{1B}$ cDNAs within the putative IVS5–IVS6 pore-forming region and the cytoplasmic tail (5015–5623 bp) were found to be identical to the previously cloned rat brain B class $\alpha_1$-subunit (rbB-1; Dubel et al., 1992), we have identified several clusters of sequence divergence from rbB-1 in other regions of the Ca channel $\alpha_{1B}$-subunit, suggesting that multiple isoforms of the N-type Ca channel are expressed in rat sympathetic neurons (Lin et al., manuscript in press; *see also* Stea et al., 1995 for studies in rat brain).

### The Significance of the Expression of Multiple Ca Channel β-Subunits

We have shown that mRNA corresponding to all four Ca channel β-subunits can be detected in rat sympathetic neurons, with the lowest degree of amplification being obtained from the $\beta_{1b}$-specific primers. In contrast, the same $\beta_{1b}$-specific primers were just as effective as $\beta_2$- and $\beta_3$-specific primers in amplifying cDNAs derived from brain mRNA. Additional experiments are clearly needed to establish the relative abundance of these various Ca channel β-subunits. The strong signals obtained with the $\beta_3$- and $\beta_4$-specific PCR primers are, however, consistent with work from Campbell's group showing that $\beta_3$ (Witcher et al., 1993) and more recently $\beta_4$ (Scott et al., 1995) Ca channel subunits can associated with the ω-CgTx GVIA-sensitive, N-type, Ca channel purified from rabbit brain.

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