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Cardiac L-type Ca^{2+} channel triggers transmitter release in PC12 cells

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Abstract

Among the various voltage-sensitive Ca^{2+} channels present in PC12 cells are the dihydropyridine (DHP)-sensitive L-channel, the ω-conotoxin (ω-CgTx)-sensitive N-channel, and an atypical ω-CgTx/DHP-insensitive Ca^{2+} channel. Depolarization-evoked Ca^{2+} entry and [^{3}H]dopamine release is mediated by L-type Ca^{2+} channels determined by the use of Ca^{2+} channel antagonists, and a single protein of 250 kDa is recognized by L-type-specific antibodies. Screening of a PC12 cDNA library revealed two types of Ca^{2+} channels which were identified by partial sequencing. A pc12-L clone displayed virtually identical sequence homology to the cardiac L-type channel. The identical sequence homology of the single alternative splicing region confirmed clone pc12-L as the rbC-I transcript, a cardiac–neuronal α1 subunit expressed in rat brain. Clone pc12-N displayed identical sequence homology to rbB-I, a neuronal α1 subunit of the N-type Ca^{2+} channel expressed in rat brain; Northern blot analysis identified RNA of a size similar to that previously described for rat brain.

Key words: cDNA cloning; Dihydropyridine receptor; N-type Ca^{2+} channel; L-type Ca^{2+} channel; PC12 cell

1. Introduction

High voltage-activated Ca^{2+} channels which regulate Ca^{2+} entry into cells are classified into three main types, L-, N- and P-channels, according to their pharmacological properties, kinetics and voltage sensitivity [1–4]. L-type Ca^{2+} channels are composed of four subunits (α1, α2/δ, β, and γ) of which α1 is responsible for Ca^{2+} entry [1–4]. Recently, immunoprecipitation studies showed a similar multisubunit complex (α1, α2/δ, β and 95K) for a functional rabbit brain N-type channel [5], suggesting that the corresponding subunits perform similar functions within the two complexes. At least three distinct genes were found to encode dihydropyridine (DHP)-sensitive L-type α1 subunits and one gene for ω-CgTx-sensitive α1 subunits of the N-type Ca^{2+} channel [6,7]. The P-type Ca^{2+} channel, which is ω-Aga toxin (ω-AgaTx)-sensitive, was first identified in cerebellum [8], and has been cloned and sequenced [9,10].

Ca^{2+} channels of the L-type can be sub-classified into three distinct families; the skeletal type, confined to skeletal muscle, the cardiac C-type, and the neuroendocrine D-type. The C-type is the most abundant channel, and is expressed in rat brain as shown by the two cDNAs, designated rbC-I and rbC-II, encoding two transcripts of the α1 subunit [11]. These two clones are closely related to rabbit cardiac [12] and lung α1 subunits [13] and display lower homology (approximately 70%) to the skeletal L-type channel [14]. The neuroendocrine D-type L-channel, which is expressed in human pancreatic β cells, bears 68% identity to the α1 subunit of rabbit heart [15]. The presence of both L- and N-type, but not the P-type, Ca^{2+} channels has been determined in neurosecretory PC12 cells by electrophysiological methods [16,17]. However, the subfamily of L-type channel in these cells has not yet been described. PCR analysis of rbB-1 (the N-type channel), showed that both non-differentiated and NGF-treated PC12 cells express N-type channels similar in size to the rbB-I product expressed in rat brain [6]. In this study, we report for the first time the cloning and identification by sequence homology, of the PC12-specific L-type α1 subunit, and identify by L-type-specific antibodies a single 250 kDa protein.

2. Materials and methods

Neonatal rat heart muscle cells were obtained from A. Pinson (Hadassa Medical School Jerusalem); ω-Conotoxin GVIA (ω-CgTx) and ω-Agatoxin IVA (ω-AgaTx) were purchased from Alomone Labs. (Jerusalem).

2.1. Cell growth

PC12 cells were kindly provided by Dr. Rick Chris (NYU, USA). The growth medium consisted of Dulbecco's modified Eagle's medium (DMEM) with high glucose (Gibco), supplemented with 10% horse serum, 5% fetal calf serum, 130 U/ml penicillin and 0.1 mg/ml streptomycin. For assays, cells were removed using 1 mM EDTA and re-plated on collagen-coated 12-well plates and assayed 24 h later.

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Abbreviations: DHP, dihydropyridine; ω-CgTx, ω-conotoxin GVIA; ω-AgaTx, ω Agatoxin IVA; [^{3}H]DA, [^{3}H]dopamine.

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2.2. [^{3}H]DA release assay

Release was determined essentially as previously described [18]. Briefly, cells were incubated for 1.5 h at 37°C with 0.5 ml growth medium and 0.85 μl [^{3}H]DA (41 Ci/mmol), and 10 μg/ml pargyline followed by extensive washings with medium (3 × 1 ml) and release buffer consisting of (mM): 130 NaCl; 5 KCl; 25 $NaHCO_3$; 1 NaH_2PO_4; 10 glucose; and 1.8 $CaCl_2$. In a typical experiment, cells were incubated with 0.5 ml buffer for five consecutive incubation periods of 3 min each at 37°C. Spontaneous [^{3}H]DA release was measured by collecting the medium released by the cells during the initial two successive 3 min periods. Antagonists were added to the cells 3 min prior to stimulation (at the second period), and stimulation (60 mM KCl) of release was monitored during the third period. The remaining [^{3}H]DA was extracted from the cells by overnight incubation with 0.5 ml 0.1 N HCl. [^{3}H]DA release during each 3 min period was expressed as a percentage of the total ^{3}H content of the cells. Net evoked release was calculated from [^{3}H]DA released during the stimulation period after subtracting basal [^{3}H]DA release in the preceding, baseline period if not indicated otherwise [18].

2.3. $^{45}CaCl_2$ influx

$^{45}Ca^{2+}$ influx in PC12 cells was assayed essentially as previously described [19]. Briefly, cells were pre-incubated for 1 h with [^{3}H]adenine (0.2 μCi/ml), washed in 2 × 0.5 ml aliquots of influx buffer consisting of (mM): 108 NaCl; 0.5 $CaCl_2$; 4.7 KCl; 1.2 $MgSO_4$; 10 glucose; and 25 HEPES, pH 7.4; and influx was stimulated by the secretagogue as indicated, in the presence of $^{45}CaCl_2$ (2 μCi/ml) for 3 min at 37°C. ^{45}Ca influx was terminated by extensive washings (× 5) of the cells with ice-cold 0.1 M $MgSO_4$. Ca^{2+} channel blockers were added to the cells 3 min prior to stimulation and were present during the 3 min stimulation period. The cells were extracted with 0.1 N SDS and the radioactivity counted. Correction for cell numbers was made by counting the [^{3}H]adenine taken up by the cells during the pre-incubation period.

2.4. Immunoblot analysis

Polyclonal antibodies (anti-CP25) were generated against a 20mer peptide of the α1 subunit of rabbit skeletal muscle (1,419–1,431 (KHLDVVTLLRRIQPPLGFGK), coupled to bovine serum albumin by De Jongh, and used as previously described [20]).

2.5. Protein determinations, SDS-PAGE and immunoblotting

Cells were solubilized in Triton X-100 and proteins were separated by SDS-PAGE. Proteins were transferred to nitrocellulose using a semi-dry electrotransfer apparatus (Pharmacia-LKB-Novablot). The blots were incubated, blocked with blocking buffer containing 0.3% Tween-20 and 5% non-fat dry milk in Tris-buffered saline (TBS) for 30 min, and than with anti-CP-25 in blocking buffer for 1 h. After five washes with 0.1% Tween 20 in TBS the blot was exposed to peroxidase-conjugated affinity-pure goat anti-rabbit IgG for 1 h, washed and detected by the enhanced chemiluminescence detection system (ECL). Protein was determined using Peterson's method [21].

2.6. Isolation of rbB-1 type cDNA clone and sequencing

The 2.7 kb fragment of the pancreatic β-cell, a gift from Dr. Seino, Japan, was cut with *Hin*dIII and *Bgl*II and a 1,730 bp fragment (4,160–5,890) was used as a probe to screen a rat λZAP II-PC12 cDNA library (a gift from Dr. J. Boulter, CA). Recombinant phages (5 × 10^5) were plated and screened with the ^{32}P-labeled probe (1 × 10^9 cpm/μg) prepared with the Multiprime DNA labeled system (Amersham, USA) using standard procedures [22]. The positive clone (1 kb) was subcloned into plasmid vector Bluescript (Startagene) by conventional techniques, and sequenced by the dideoxy chain termination method using an Applied Biosystem 373DNA sequencer.

2.7. Isolation of rbC type cDNA clone and cDNA sequencing

The complete cDNA sequence of rbC-II, a gift from Dr. T. Snutch (Canada) was cut by *Eco*RI, radiolabeled by random priming (see above), and used to screen 5 × 10^5 recombinants of a rat cDNA library (λgt11; Clontech Labs Inc., USA). A positive clone (2.3 kb) was isolated, purified on gel, subcloned into pBluescript and sequenced (see above).

2.8. Northern blot analysis

Total RNA was isolated from PC12 cells, homogenized in 5 M guanidinium thiocyanate followed by precipitating RNA through a CsCl gradient [22]. mRNA was isolated with an oligo dT column, applied to a 1.1% agarose gel containing 1.1 M formaldehyde, and transferred to Hybond-N nylon membrane. Hybridization was done as described [22] by using a pc12-N clone that had been radiolabeled with [α-^{32}P]dCTP by random priming.

3. Results and discussion

3.1. What type of Ca^{2+} channel is responsible for transmitter release in PC12 cells?

The presence of both L- and N-type channels in PC12 cells was determined by voltage-clamped measurements

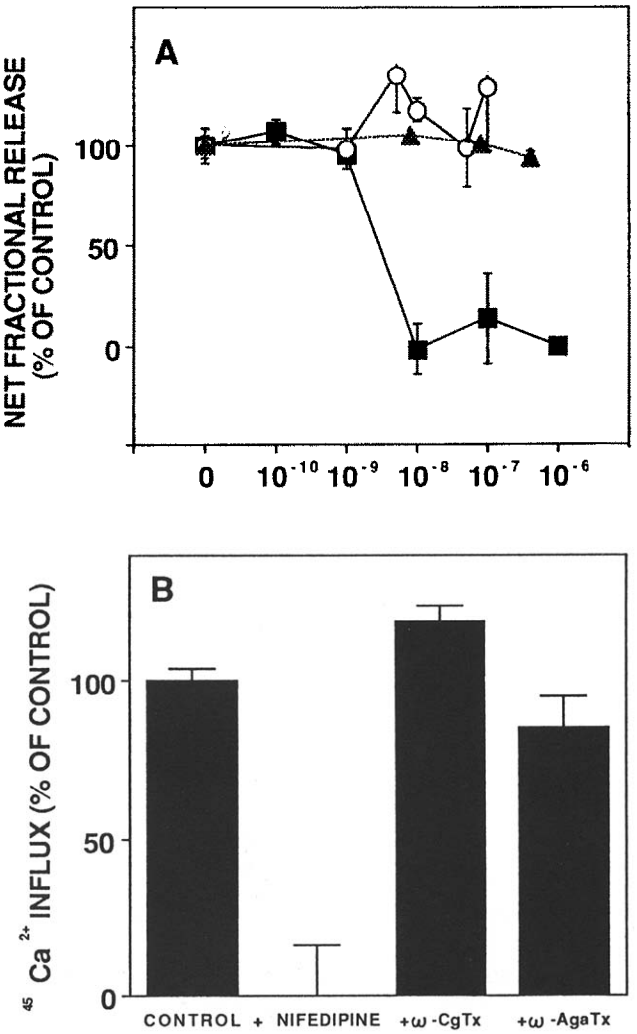

Fig. 1. Inhibition of depolarization induced [^{3}H]DA release and $^{45}Ca^{2+}$ influx in PC12 cells. (A) Depolarization-induced [^{3}H]DA release in PC12 cells was carried out according to the procedure described in section 2. [^{3}H]DA released in response to a 3 min stimulation by 60 mM KCl at 37°C in the presence of ω-CgTx (○), ω-AgaTx (▲) and nifedipine (■), at increasing concentrations, as indicated. Data are presented as net fractional release (see section 2). (B) Depolarization-induced $^{45}Ca^{2+}$ influx in PC12 cells was carried out as described in section 2. Data are presented as net $^{45}Ca^{2+}$ influx stimulated for 3 min in 10^5 cells, 37°C, by 60 mM KCl alone (control) and in the presence of nifedipine (1 μM), ω-CgTx (1 μM), and ω-AgaTx (0.5 μM).

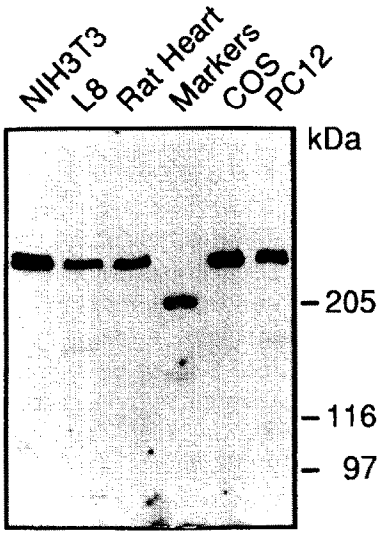

Fig. 2. Immunoblotting of the α1 subunit of voltage-sensitive L-type Ca^{2+} channels in extracts of NIH 3T3, L8, rat heart primary COS and PC12 cells. The samples (100 μg protein) were separated by gel electrophoresis using a 5% gel and then electrotransferred to nitrocellulose. Immunoblotting was carried out with anti-CP-25 (see section 2). Numbers on the right indicate positions of molecular weight markers. One of the Rainbow markers (Sigma, USA) is recognized by the second antibody (lane 4).

of undifferentiated [16] and differentiated [17] PC12 cells, and by PCR amplification of clone rbB-I [6].

Transmitter release and $^{45}Ca^{2+}$ influx stimulated by membrane depolarization were completely abolished by 10 nM nifedipine (Fig. 1A,B), excluding the possible involvement of other L-type-activated channels in the release process. Similar results were previously reported for KCl-induced [^{3}H]norepinephrine release in undifferentiated PC12 cells [23].

In contrast to presynaptic currents generated by P-type Ca^{2+} channels, which induce transmitter release at the mouse neuromuscular junction and brain synaptosomes [24], the highly specific toxin for P-type Ca^{2+} channels, ω-AgaTx (0.5 μM), did not affect depolarization-induced transmitter release or $^{45}Ca^{2+}$ influx (Fig. 1A,B). Similarly, N-type Ca^{2+} channels, which are concentrated at the presynaptic membranes of nerve terminals, are proposed to be closely associated with synaptic transmission [25,26]. Nevertheless, evoked transmitter release or calcium influx were insensitive to ω-CgTx (0.1 μM; Fig. 1A,B).

3.2. Characterization of the L-type α1 subunit in PC12 cells

Anti-CP-25, which was raised against the L-type-specific sequence of rabbit skeletal muscle α1 subunit [20] was used for detecting expression of the L-type α1 subunit in PC12 cells. According to the immunoblot presented, a single protein of 250 kDa was recognized by anti-CP-25. Neither the rat brain α1 subunit, Rb1 (1,646 amino acids [27]), nor the rabbit skeletal muscle (1,873 amino acids 212 kDa), were detected ([28] Fig. 2). NIH 3T3, L8, neonatal rat heart muscle cells and COS cell extracts were similarly treated for comparison. The recognized protein expressed in PC12 cells was identical in size to the α1 subunit present in cardiac neonatal cells and in the other cell extracts. Further studies should determine whether this high molecular weight α1 subunit is the cardiac subclass C (242 kDa or one of its alternative transcripts), or the neuroendocrine subclass D (247 kDa), since both products are similar in size (Fig. 2).

3.3. The L-type Ca^{2+} channel expressed in PC12 cells

Further data on the L-type channel which is expressed in PC12 cells was obtained from a partial sequence of pc12-L, a 2.3 kb clone, isolated by screening of a λgt11 cDNA library. Sequencing of ~ 1,000 bp showed virtually 100% sequence homology to the cardiac L-type

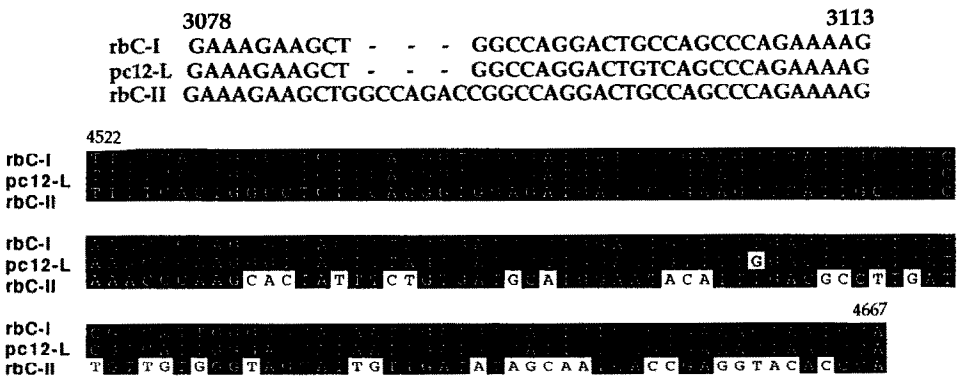

Fig. 3. (A) Nucleotide alignment of a 9 bp deletion region in the α1 subunit of C-type Ca^{2+} channel transcripts expressed in rat brain [11] and in PC12 cells. Nucleotide numbers are from the rbC-I DNA sequence, accession number M67156. (B) Sequence alignment of an alternative splicing region at the α1 subunit of rat brain rbC-I and rbC-II and pc12-L. The region covers the alternative splicing region which is at the IVS3 domain (region of the third transmembrane segment of domain IV of the α1 subunit). The black areas represent sequence homology. Nucleotide numbers are from the rbC-I DNA sequence, accession number M67156.

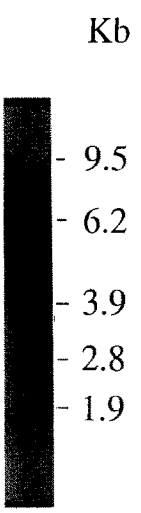

Fig. 4. Northern blot analysis of pc12-N expression in PC12 cells. Autoradiograph of a blot hybridization of a 1 Kb cDNA probe isolated from a λZAP cDNA library (pc12-N). The size of the transcript was determined using RNA standards (Promega). Autoradiography was for 3 days with intensifying screens.

channel. Two variants of the cardiac L-type channel (designated rbC-I and rbC-II) which are formed by a single alternative splicing at the S3 of domain IV, and encoded by the same gene, have been previously cloned from rat brain [11].

These two variants differ in three amino acid deletions (P, A, R), in the cytoplasmic loop separating domains II and III, and 13 single amino acid substitutions in a stretch of 28 amino acids at the S3 segment of domain IV, shown to be an alternative splicing region [11]. The rbC-I transcript is highly expressed in the adrenal and pituitary gland [12], and is 95% homologous to the cardiac DHP-sensitive Ca^{2+} channel from rabbit. As shown in Fig. 3A,B, both the PAR deletion region and the single alternative splicing region showed 100% homology to rbC-I, establishing pc12-L as an rbC-I transcript. Although the rbC-II transcript or the D-subtype were not found in our screening, their expression cannot be excluded.

3.4. *Partial sequence of the N-type Ca^{2+} channel of PC12 cells and its full size cDNA*

A *Hin*dIII–*Bgl*II fragment (4,160–5,890 bp according to rbB-I; accession number M92505), excised from 2.7 Kb cDNA, codes for the C-terminal region of the α1 subunit of the voltage-dependent Ca^{2+} channel that is expressed in pancreatic β cells [15]. This fragment was used as a probe to screen a cDNA library derived from PC12 cells [22]. A partial sequence (690 bp) corresponding to the two ends of the isolated 1 kb clone (pc12-N), turned out to be virtually identical (>99%) to the α1 subunit of the N-type Ca^{2+} channel, rbB-I, cloned from rat brain [6], and > 90% identity with the human neuronal Ca^{2+} channel α1 subunit ($\alpha_{1B\text{-}1}$; [7]). Complete identification of the N-type channel expressed in PC12 cells will require additional sequencing. Northern blot analysis using clone pc12-N revealed a message of similar size to rat brain [6] and an additional band which could be another transcript of a lower size (Fig. 4). In analogy to chromaffin cells, the N-channel in PC12 cells is not functional (shown in Fig. 1A). Chromaffin cells contain ω-CgTx binding sites [29,30] and ω-CgTx-sensitive currents [31,32] but neither catecholamine secretion nor increase of $[Ca^{2+}]_i$ transients were antagonized by ω-CgTx [33,34]. Furthermore, synaptotagmin, a small synaptic vesicle-associated protein which is involved in mediating transmitter release [35], is absolutely non-essential in the secretion process in PC12 cells [36]. This result led to the suggestion that synaptotagmin is associated only with ω-CgTx-sensitive Ca^{2+} channels [36], which indeed, as shown in the present study, do not participate in DA release in PC12 cells. It may be concluded that transmitter release in PC12 cells is mediated solely by L-type Ca^{2+} channels of the cardiac C-type, most likely by the rbC-I subtype. Recently it was shown that three types of Ca^{2+} channels trigger secretion in chromaffin cells of which the major contributor is the L-type facilitation channel [37]. PC12 cells might have a lower expression of N- and P-channels compared to chromaffin cells and, if their coupling efficiency is low, as shown for chromaffin cells, their contribution to induced secretion would be undetected.

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