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Molecular cloning of a murine N-type calcium channel α1 subunit

Evidence for isoforms, brain distribution, and chromosomal localization

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Abstract

A cDNA encoding a N-type Ca^{2+} channel has been cloned from the murine neuroblastoma cell line N1A103. The open reading frame encodes a protein of 2,289 amino acids (257 kDa). Analysis of different clones provided evidence for the existence of distinct isoforms of N-type channels. High levels of mRNA were found in the pyramidal cell layers CA1, CA2 and CA3 of the hippocampus, in the dentate gyrus, in the cortex layers 2 and 4, in the subiculum and the habenula. The N-type Ca^{2+} channel gene has been localized on the chromosome 2, band A.

Key words: Calcium channel; Brain; Isoform; Murine chromosomal localization

1. Introduction

Voltage-dependent Ca^{2+} channels regulate Ca^{2+} entry and thereby contribute to Ca^{2+} signalling in many cells. The different types of Ca^{2+} channels (L-, N-, P-, and T-types) were originally defined based on their biophysical properties and on their sensitivities towards drugs and divalent ions [1]. More recently, molecular cloning revealed an even greater diversity amongst voltage-dependent Ca^{2+} channels. At least six different genes have been identified [2–4], and an even greater functional variety might be produced by alternate splicing of the different gene products. The N-type Ca^{2+} channel which is inhibited by ω-Conotoxin GVIA (ωCgTx) plays an important role in neuronal tissues [5,6]. For example ωCgTx inhibits the secretion of neurotransmitters stimulated by depolarisation [7,8]. Biochemical studies have indicated that ωCgTx-binding sites are predominantly present in neuronal tissues and have identified the ωCgTx-receptor as a protein of 210–220 kDa [9].

Here we report the molecular cloning of a murine N-type Ca^{2+} channel, the presence of isoforms, the distribution of its RNA in mouse brain and the localization of the gene.

2. Materials and methods

2.1 Tissue culture, RNA preparation and Northern hybridization

The neuroblastoma cell line N1A103 was cultured in DMEM supplemented with 10% foetal calf serum and used for RNA preparation at 50% confluency. Total RNA was extracted by the guanidinium thiocyanate method [10]. Poly(A)$^+$RNA was purified on an oligo(dT) cellulose column equilibrated with 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 M NaCl, 0.1% SDS, eluted with water, and precipitated. Northern blots were performed as described [11] using an ^{32}P-labeled *Apa*I fragment (nucleotides 2,761–3,203) as probe.

2.2. PCR amplification

Primers used in this work corresponded to regions conserved in different cloned Ca^{2+} channels. The sense primer (5′-ATGGARGGNTGGACNGA-3′) corresponded to a sequence located between transmembrane segment IS5 and IS6 (MEGWTD), which is conserved in all mammalian voltage-dependent Ca^{2+} channels cloned so far, except the low voltage-activated Ca^{2+} channel [12]. The antisense primer (5′-CCNCCRAANAGYTGCAT-3′) was designed according to a sequence located on the hydrophobic segment IIS5 (MQLFGG), which is conserved in all cloned voltage-gated Ca^{2+} and Na^+ channels. PCR amplifications were performed with 10 ng of cDNA from N1A103 cells, 100 ng of each primer, 200 μM dNTPs and 4 U *Taq* polymerase in 50 μl 1 × *Taq* buffer (1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.3, 0.1 mg/ml gelatin) using the following protocol: 30 s at 94°C, 1 min at 57°C and 1 min at 72°C. The product was subcloned in the pBluescript SKII(+) vector and sequenced on both strands by dideoxy sequencing using the dye terminator kit and automatic sequencing (Applied Biosystem 373A). PCR amplifications for the verification of splice variants were carried out using the same conditions.

2.3. cDNA library synthesis and screening

Two oligo(dT) primed cDNA libraries in λ-ZapII (Stratagene) were prepared from the N1A103 mRNA using the technique of Gubler and Hoffman for cDNA synthesis and standard cloning techniques [13]. 10^6

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recombinants of each library were screened with the PCR product radiolabelled with [α-^{32}P]CTP by random priming (Amersham multiprime). Hybridization and washing was carried out at high stringency.

2.4. In situ hybridization histochemistry

Brain sections of adult mice were hybridized with a sense or antisense cRNA probe corresponding to an *ApaI* fragment of Nb1 (nucleotides 2,761–3,203) which was labelled with [^{35}S]UTP (1,000 Ci/mmol, Amersham). Hybridization was performed during 16 h at 60°C as described [14]. Following hybridization, the slides were washed in 1 × SSC, 10 mM 2-mercaptoethanol for 30 min at room temperature and then during 45 min at 68°C. The slices were treated for 15 min at 37°C in 2 × SSC, RNAse A (10 mg/ml), and for 30 min at 45°C in 0.1 × SSC and exposed to β-max hyperfilm (Amersham) at 4°C for 6–10 days.

2.5. Chromosomal localization

In situ hybridization experiments were carried out using metaphase spreads from WMP male mouse, in which all the autosomes except 19 were in the form of metacentric robertsonian translocations.

Lymphocytes were stimulated with concanavalin A and cultured at 37°C for 72 h. 5-Bromodeoxyuridine was added for the final 6 h of culture (60 μg/ml of medium), to ensure a chromosomal R-banding of good quality.

An insert of 800 base pairs (nucleotides 2,577–3,386) in pBluescript SKII(+) was tritium-labelled by nick-translation to a specific activity of 1.7 10^8 dpm/μg. The radiolabelled probe was hybridized to metaphase spreads as described previously [15].

After coating with nuclear track emulsion (Kodak NTB2), the slides were exposed for 15 days at +4°C. To avoid any slipping of silver grains during the banding procedure, chromosome spreads were first stained with buffered giemsa solution and metaphases photographed. R-banding was performed by the fluorochrome-photolysis-giemsa (F.P.G.) method and metaphases reprographed before analysis.

3. Results and discussion

Fig. 1 shows the deduced amino acid sequence of the Ca^{2+} channel (Nb1) from the mouse neuroblastoma (N1A103) cell line. The clones we isolated contained an open frame of 2,289 amino acids (M_r = 256,590). The nucleotides surrounding the first in frame ATG (5′-GGAGTCATGG-3′) agree reasonably well with the consensus sequence for translation initiation [16]. The sequence Nb1 is similar to the previously reported class B gene product from rat brain (Rb-B; 95% amino acid sequence identity) [17], from human neuroblastoma cells (α1B; 94% amino acid sequence identity) [18] and from rabbit brain (BIII; 95% amino acid sequence identity) [19]. Thus, the Nb1 channel appears to be a mouse counterpart of these rat, human and rabbit channels, corresponding to a N-type Ca^{2+} channel.

The Nb1 Ca^{2+} channel displays general structural features common with the other voltage-dependent Ca^{2+} channels [20] in that it consists of four repeated units of homology. Each motif has five hydrophobic segments (S1, S2, S3, S5 and S6) and one positively charged segment (S4), which is thought to represent the voltage-sensor [21]. The charged residues in segments S2 and S3 are also conserved. The Nb1 channel has three potential N-glycosylation sites (Asn^{256}, Asn^{1551} and Asn^{1664}) on the extracellular side. It has 2 potential phosphorylation sites for protein kinase A, and 18 for protein kinase C on the cytoplasmic side. A consensus sequence for ATP binding ('P-loop') [22] is positioned between amino acid residues 457 and 465 (**ASLKSGKT**).

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MVRFGDELGGRYGGTGGGERARGGGAGGAGGPGQGGLPPGQRVLYKQSIAQRARTMALYN 60
PIPVKQNCFTVNRSLFVFSEDNVVRKYAKRITEWPPFEYMILATIIANCIVLALEQHLPD 120
GDKTPMSERLDDTEPYFIGIFCFEAGIKIIALGFVFHKGSYLRNGWNVMDFVVVLTGILA 180
TAGTDFDLRTLRAVRVLRPLKLVSGIPSLQVVLKSIMKAMVPLLQIGLLLFFAILMFGII 240
GLEFYMGKFHKACFPNSTDTEPVGDFPCGKDPPARQCDGDTECREYWPGPNFGITNFDNI 300
LFAILTVFQCITMEGWTDILYNTNDAAGNTWNWLYFIPLIIIGSFFMLNLVLGVLSGEFA 360
KERERVENRRAFLKLRRQQQIERELNGYLEWIFKAEEVMLAEEDKNAEEKSPLDVLKRAA 420
TKKSRNDLIHAEEGEDRFVDLCAVGSPFARASLKSGKTESSSYFRRKEKMFRFFIRRMVK 480
AQSFYWVVLCVVALNTLCVAMVHYNQPQRLTTALYFAEFVFLGLFLTEMSLKMYGLGPRS 540
YFRSSFNCFDFGVIVGSIFEVVWAAIKPGTSFGISVLRALRLLRIFKVTKYWNSLRNLVV 600
SLLNSMKSIISLLFLLFLFIVVFALLGMQLFGGQFNFQDETPTTIFDTFPAAILTVFQIL 660
TGEDWNAVMYHGIESQGGVSKGMFSSFYFIVLTLFGNYTLLNVFLAIAVDNLANAQELTK 720
DEEEMEEAANQKLALQKAKEVAEVSPMSAANISIAAQQNSAKARSVWEQRASQLRLQNLR 780
ASCEALYSEMDPEERLRYASTRHVRPDMKTHMDRPLVVEPGRDGLRGPVGSKSKPEGTEA 840
TESADLPRRHHRHRDRDKTSATAPAGGEQDRTESTETGPREERARPRRSHSKETPGADTQ 900
VRCERSRRHHRRGSPEEATEREPRCHRAHRHAQDSSKEGTVPVLVPKGERRARHRGPRTG 960
PREAENNEEPTRRHRARHKVPPTLQPPERFAAEKESNPVEGDKETRNHQPKEPHCDLEAI 1020
AVTDVGPLHMLPSTCLQKVDEQPEDADNQRNVTRMGSQPSDPSTTVHVPVTLTGPPGETP 1080
VVPSGNMNLEGQAEGKKEAEADDVLRRGPRPIVPYSSMFCLSPTNLFRRFCHYIVTMRYL 1140
EMVILVVIALSSIALAAEDPVRTDSFRNNALEYMDYIFTGVFTCEMVIKMIDLGLLLHPG 1200
AYFRDLWNILDFIVVSGALVAFAESGSKGKDINTIKSLRVLRVLRPLKTIKRLPKLKPVF 1260
DSVVNSLKNVLNILIVYMLFMFIFAVIAVQLFKGKFFYCTDESKELERDCRGQYLDYEKE 1320
EVEAQPRQWKKYDFHYDNVLWALLTLFTVSTGEGWPMVLKHSVDATYEEQGPSPGFRMEL 1380
SILYVVYFVVFPFFFVNIFVALIIITFQEQGDKVMSECSLEKNERACIDFAISAKPLTRY 1440
MPQNKQSFQYKTWTFVVSPPFEYFIMAMIALNTVVLMMKFYDAPYEYELMLKCLNIVFTS 1500
MFSMECILKIIAFGVLNYFRDAWNVFDFVTVLGSITDILVTEIAETNNFINLSFLRLFRA 1560
ARLIKLLRQGYTIRILLWTFVQSFKALPYVCLLIAMLFFIYAIIGMQVFGNSALDDDTSI 1620
NRHNNFRTFLQAIMLLFRSATGEAWHEIMLSCLDNRACDPHANASECGSDFAYFYFVSFI 1680
FLCSFLMLNLFVAVIMDNFEYLTRDSSILGPHHLDEFIRVWAEYDPAACGRISYNDMFEM 1740
LKHMSPPLGLGKKCPARVAYKRLVRMNMPISNEDMTVHFTSTLMALIRTALEIKLAPADE 1800
MTVGKVYAALMIFDFYKQNKTTRDQTHQAPGGLSQMGPVSLFHPLKATLEQTQPAVLRGA 1860
RVFLRQKSATSLSNGGAIQTQESGSRSRCPGGRRGTQDALYLGRAPLLRDHSKEIPVGQS 1920
GTLLVDVQMQNMTLRGPDGDPQPGLESQGRAASMLRLAAETQPAPNASPMKRSISTLAPR 1980
PDGTQLCSTVLDRPPPSQASHHHHHRCHRRRDKKQRSLEKGPSLSVDPEGAPSTAAAGPG 2040
LPHGEGSTACRRDRKQERGRSQERRQPSSSSSEKQRFYSCDRLGAGSPQLMPSLSSHPTS 2100
PAAALEPAPHPQGSGSVNGSPLMSTSGAITPGRGGRRQLPQTPLTPRPSITYKTANSSPV 2160
HFAEGQSGLPAFSPGRLSRGLSEHNALLQKEPLSQPLAPGSRIGSDPYLGQRLDSEASAH 2220
TLPEDTLTFEEAVATNSGRSSRTSYVSSLTSQSHPLRRVPNGYHCTLGLNTGVGARASYH 2280
HPDQDHWC*                                                      2289
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Fig. 1. Deduced amino acid sequence of the Nb1 Ca^{2+} channel. The nucleotide sequence of Nb1 was determined using clone NPS2 (nucleotides 1 to 1,437) obtained from the specifically primed library and dt1 (1,110 to 7,351) from the oligo dT primed library. The amino acid sequence shown corresponds to an open reading frame between the first in frame ATG at nucleotide 121 (which was preceded by an in frame stop codon) and the stop codon at nucleotide 6,984. The putative transmembrane segments S1-S6 in each of the repeats I-IV are underlined. (○) N-glycosylation site; (+) protein kinase C site; (*) protein kinase A site; bold residues ATP and GTP binding consensus sequence.

Besides the clone whose sequence is shown in Fig. 1, we identified additional clones that show differences in some regions (Fig. 2A). An insertion of one amino acid (Ala^{414}), between the first and second transmembrane domain, was found in one of the clones obtained from the oligo(dT)-primed library and in one overlapping clone obtained from the specifically primed library (Fig. 2A). This difference together with the fact that the same amino acid is also lacking in the human sequence, while it is present in the rat sequence, suggests that the two forms are in fact coexisting in the neuroblastoma cells N1A103. This difference of one amino acid might be due to an allelic polymorphism.

On the oligo(dT)-primed clone (λdT2) which contains the insertion of Ala^{414}, we identified an additional insertion in the cytoplasmic loop between transmembrane domains IIS6 and IIIS1. This additional sequence of 22 amino acids, particularly rich in serine residues (7 out of 22 amino acids), introduces one potential phosphorylation site for Ca^{2+} calmodulin-dependent protein kinase II and one for protein kinase C. This insertion has also been confirmed by PCR using primers surrounding this deletion (Fig. 2B). Alternate splicing of L-type Ca^{2+} channels was shown to be tissue-specific and development-dependent [23] and it might hence be a mechanism to fine tune Ca^{2+} channel function between tissues and during development.

For the L-type Ca^{2+} channel from skeletal muscle, the cytoplasmic loop between transmembrane domains IIS6 and IIIS1 has been shown to be crucial for excitation contraction coupling [24]. The N-type Ca^{2+} channel has a much larger intracellular loop. It is tempting to speculate that it might be involved in processes like excitation to secretion coupling.

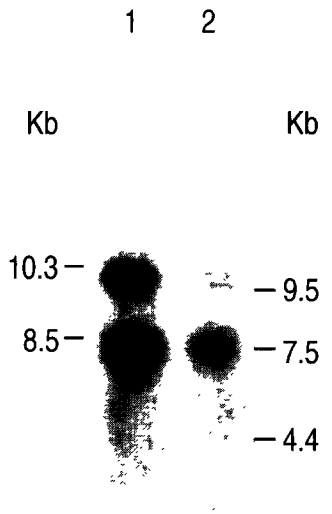

Fig. 3. Northern blot analysis of the Nb1 RNA expression in mouse brain and N1A103 neuroblastoma cells. Northern blot analysis of poly(A)$^+$ RNA (2 μg each) from mouse brain (lane 1) and the neuroblastoma cell line N1A103 (lane 2) using a 441 bp *Apa*I fragment corresponding to amino acids 879–1,026, a part of the cytoplasmic loop as probe. Autoradiography was at −70°C for 8 days with an intensifying screen. A RNA ladder (Bethesda Research Laboratories, Gaithersburg, MD) was used as size marker.

3.1. Tissue distribution

The size and tissue distribution of the Nb1 RNA was examined by Northern hybridization. Amongst the tissues we examined, a signal was observed for the N-type Ca^{2+} channel only in brain and in neuroblastoma cells. There are two forms of the Nb1 mRNA (8.5 kb and 10.3 kb) present in brain as well as in the N1A103 cells

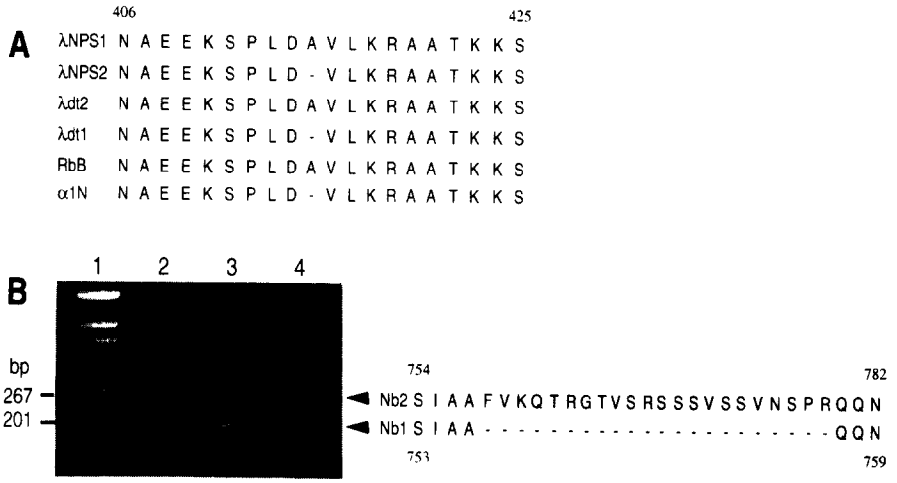

Fig. 2. (A) Partial alignment of the amino acid sequence between transmembrane domains I and II. NPS1 and NPS2: clones obtained from a specifically primed library; λdt1 and λdt2 clones obtained from an oligo(dT)-primed library; RbB rat N-type Ca^{2+} channel [17], and α1N human N-type Ca^{2+} channel [18]. The numbers on the top of the alignment correspond to the position in Nb1. (B) Splicing within the cytoplasmic loop between transmembrane domains II and III. PCR experiments werre carried out using sense primer 5′-GAAGAGGAGATGGAAGAA-3′ (position 2,283–2,301) and anti sense primer 5′-ACTGTACAATGCCTCACA-3′ (position 2,484–2,466). Lane 1 molecular weight markers, lane 2 N1A103 mRNA not reverse transcribed, lane 3 cDNA from mouse brain, lane 4 cDNA from N1A103. The expected sizes for the amplification products are 201 bp for Nb1 and 267 bp for the variant Nb2.

(Fig. 3). The cDNA we cloned presumably corresponds to the lower band.

3.2. mRNA distribution in the mouse brain

In situ hybridization histochemistry of the Nb1 channel revealed widespread but uneven signals throughout the mouse brain (Fig. 4). The Nb1 mRNA was detectable in the olfactory bulb (OB) (the external and internal plexiform layers and the mitral and granular cell layers), anterior olfactory cortex, olfactory tubercule, caudate-putamen, primary olfactory cortex, neocortex, enthorhinal cortex, hippocampal formation, amygdaloid nucleus, thalamus-hypothalamus, colliculus, cerebellar cortex, and medulla-pons (the motor trigeminal nucleus, facial nucleus, lateral reticular nucleus, and inferior olivary nucleus). The regions expressing the highest levels of Nb1 mRNA (Fig. 4A) were: the pyramidal cell layers CA1, CA2 and CA3 of the hippocampus (Hip); the dentate gyrus (DG); the cortex layers 2 and 4 (Cx); the subiculum (Sb); the habenula (Hb). Striatum and hypothalamus were less intensively labelled with the Nb1 probe. Autoradiography of coronal sections hybridized with Nb1 cRNA probes present evidence for expression of Nb1 mRNA in the granular cells (GC) of the cerebellum (Fig. 4C). The labelling corresponds to regions rich in neuronal connections. This localization is globally in agreement with the distribution of the ωCgTx receptor [25] and with immunohistological studies [26].

3.3. Chromosomal localization of Nb1 on the mouse genome

Because the N-type Ca^{2+} channel is essential for neuronal function and because its corresponding gene might be the target of mutations that cause diseases, it was important to make a chromosomal localization.

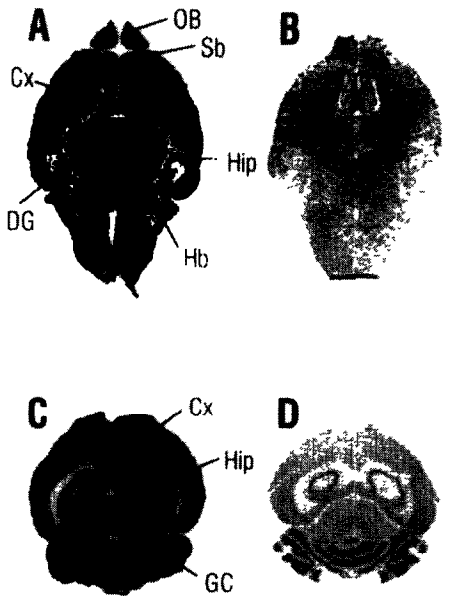

Fig. 4. Spatial distribution of Nb1 Ca^{2+} channel mRNAs determined by in situ hybridization. (A,B) Transversal section. (C,D) Coronal section. Slices were hybridized with ^{35}S-labelled Nb1 cRNA probes (antisense A and C, sense B and D).

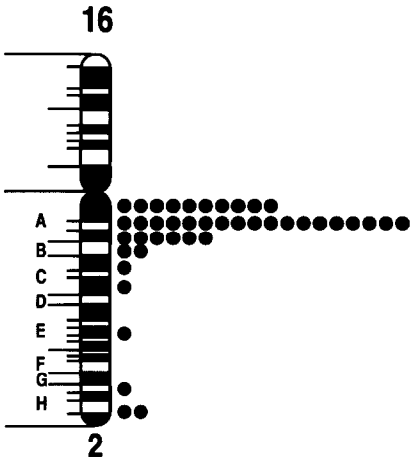

Fig. 5. Localization of the NbCaN gene to mouse chromosome 2 by in situ hybridization. Diagram of WMP mouse Rb (2; 16) chromosome, indicating the distribution of labelled sites on chromosome 2.

The Nb1 gene was localized on the murine genome by in situ hybridization. In the 100 metaphase cells examined there were 211 silver grains associated with chromosomes and 42 of these (19.9%) were located on chromosome 2. The distribution of grains on this chromosome was not random: 34/42, 80.9% of them mapped to the A band of chromosome 2 (Fig. 5). These results allow us to map the Nb1 Ca^{2+} channel probe to the 2A band of the murine genome.

A gene coding for a L-type dihydropyridine-sensitive Ca^{2+} channel was previously identified on mouse chromosome 1 [27].

The molecular cloning of the murine N-type Ca^{2+} channel was important for further investigations of the role of this channel in neuronal tissue using transgenase. Knocking out the N-type Ca^{2+} channel gene in mice should provide important information on the exact function of the channel in brain activity and development.

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