Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 5058–5062, June 1992
Physiology

Molecular cloning of the α-1 subunit of an ω-conotoxin-sensitive calcium channel

(cDNA/immunoprecipitation/dihydropyridine receptor/expression)

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Contributed by William A. Catterall, February 12, 1992

ABSTRACT **Of the four major types of Ca channel described in vertebrate cells (designated T, L, N, and P), N-type Ca channels are unique in that they are found specifically in neurons, have been correlated with control of neurotransmitter release, and are blocked by ω-conotoxin, a neuropeptide toxin isolated from the marine snail *Conus geographus*. A set of overlapping cDNA clones were isolated and found to encode a Ca channel α-1 subunit, designated rbB-I. Polyclonal antiserum generated against a peptide from the rbB-I sequence selectively immunoprecipitates high-affinity ^{125}I-labeled ω-conotoxin-binding sites from labeled rat forebrain membranes. PCR analysis shows that, like N-type Ca channels, expression of rbB-I is limited to the nervous system and neuronally derived cell lines. This brain Ca channel may mediate the ω-conotoxin-sensitive Ca influx required for neurotransmitter release at many synapses.**

Calcium entry into cells through voltage-gated Ca channels mediates a wide variety of cellular and physiological functions. Electrophysiological and pharmacological criteria have defined four main types of Ca channel in vertebrate cells (T, L, N, and P; for reviews see refs. 1 and 2). Of these, N-type Ca channels are distinct in that they have only been described in neurons and are blocked by the neuropeptide toxin, ω-conotoxin GVIA (ω-CgTx; refs. 1–7). The correlation between ω-CgTx-sensitive N-type Ca channels and the sensitivity of neurotransmitter release to ω-CgTx (8–11) has led to the proposal that N-type Ca channels are concentrated at presynaptic nerve termini and that they play a major role in mediating chemical synaptic transmission (12). Furthermore, ω-CgTx-sensitive N-type Ca channels are a target for modulation by neurotransmitters and neuropeptides (13–16) and have also been implicated as targets of autoantibodies in small cell lung carcinoma associated with Lambert–Eaton myasthenic syndrome (17, 18).

While biochemical studies show that the skeletal muscle dihydropyridine (DHP)-sensitive L-type Ca channel is composed of five distinct subunits (α-1, α-2, β, γ, and δ; for reviews see refs. 19 and 20), functional studies demonstrate that the α-1 subunit alone is sufficient to form functional voltage-gated Ca channels (21–24). In both *Xenopus* oocyte and mammalian cell expression systems the other subunits of the skeletal muscle Ca channel modulate the electrophysiological properties of L-type Ca channels (25–28). Recent studies show that the ω-CgTx receptor from brain is also a multisubunit complex that includes components homologous to the skeletal muscle L-type Ca channel (29–31).

Molecular cloning experiments show that rat brain expresses at least four major classes of Ca channel α-1 subunit (designated rbA, rbB, rbC, and rbD; ref. 32). The rbC and rbD isoforms are most closely related to DHP-sensitive Ca channels from various tissues (21, 22, 33–36), suggesting that the brain expresses two distinct forms of L-type Ca channel. However, the rbA and rbB isoforms are only moderately related to L-type Ca channels and are, therefore, of particular interest with respect to identifying Ca channels that mediate specific functions in neurons. We report the primary structure of the rbB-I Ca channel α-1 subunit¶ and show that a polyclonal antiserum against this protein selectively immunoprecipitates ^{125}I-labeled ω-conotoxin-binding sites from rat forebrain. Expression of rbB-I is limited to the rat nervous system and to cell lines that express N-type Ca channels.

EXPERIMENTAL PROCEDURES

Isolation of rbB cDNAs. We previously isolated 10 partial rat brain cDNAs that were designated rbB-type Ca channels (32). To isolate further rbB cDNAs, oligonucleotides were synthesized against the 5′ region of the rbB-10 sequence and used to screen 600,000 plaque-forming units of a size-selected rat brain cDNA library (32). The complete DNA sequence of five overlapping rbB cDNAs was determined for this study: rbB-1214 [5267 base pairs (bp)], rbB-1274 (5620 bp), rbB-10 (4789 bp), rbB-8 (7463 bp), and rbB-79 (5079 bp).

Polyclonal Antisera Production. New Zealand White rabbits were immunized with peptides: rbA-I (residues 865–881; ref. 37) KYPSSPERAPGREGPYGRE; rbB-I (residues 851–867) KYRHHRHRDRDKTSASTPA; and rbC-II (residues 821–838; ref. 38) KYTTKINMDDLQPSENEDKS to yield the antisera CNA 1, CNB 1, and CNC 1 as described (39). The amino terminal K and Y residues of each peptide are not part of the corresponding Ca channel sequence and were added for coupling and radiolabeling purposes.

Radiolabeling Brain Membranes and Immunoprecipitation. For immunoprecipitation one rat forebrain was homogenized in 15 ml of 320 mM sucrose containing 0.2 mM phenylmethylsulfonyl fluoride, pepstatin A at 1 μg/ml, and leupeptin at 1 μg/ml at 4°C. The homogenate was spun for 2 min at 5000 rpm (Sorval SS34 rotor), and the supernatant was collected. For each test 40 μg of protein was labeled with 70 nCi (2.6 kBq; 32 fmol) of ^{125}I-labeled ω-CgTx GVIA, and 200 μg of protein was labeled with 100 nCi (7.4 kBq; 1.2 pmol) of [^{3}H]PN200-100 (90 min, 4°C). The samples were diluted with a 5-fold amount of phosphate-buffered saline (PBS). Membranes were collected by centrifugation at 50,000 rpm (Beck-

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Abbreviations: DHP, dihydropyridine; NGF, nerve growth factor; ω-CgTx, ω-conotoxin GVIA.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. M92905).

man 7STi rotor) for 30 min and solubilized with 1% digitonin/300 mM KCl/PBS. Insoluble material was pelleted [50,000 rpm (Beckman 7STi rotor) 30 min], and the supernatant was added to 500 μl of PBS/0.1% digitonin. The samples were incubated for 4 hr with 5 mg of protein A-Sepharose that had previously been incubated with 40 μl of the indicated antiserum in 500 μl of PBS for 1 hr and washed three times with PBS. The protein A-Sepharose was collected by centrifugation and washed three times with 0.1% digitonin/PBS. The amount of receptor-bound ^{125}I-labeled ω-CgTx or [^{3}H]PN200-110 immunoprecipitated was determined by either γ or scintillation counting. For competition experiments, 2 pmol of either rbB or rbC peptide was added to the protein A–Sepharose–antibody complex 1 hr before the solubilized Ca channel fraction was added.

RNA Blot and PCR. Total cellular RNA was isolated from male adult rat tissues and cell lines as described (38). For Northern (RNA) blots 30 μg of total RNA per lane was electrophoresed through a 1.1% agarose gel containing 1.1 M formaldehyde and then transferred to Hybond-N nylon membrane by capillary blot. Hybridization was done as described (38) by using a 2.5-kilobase (kb) *Sst* II fragment of rbB-79 that had been gel purified and radiolabeled with [α-^{32}P]dCTP by random priming. For PCR analysis of rbB expression first-strand cDNA was synthesized from 1 μg of total RNA by using an antisense oligonucleotide specific for the 3′-noncoding region of the rbB-I cDNA (5′-GCTCA-GACCTCCTAAGAA-3′). Subsequently, an upstream sense primer (5′-GGATCACTGGTGCTAGCTG-3′) was used in conjunction with the antisense primer to amplify the cDNA for 35 cycles (1 min at 95°C, 30 sec at 52°C, 1.5 min at 72°C). The PCR products were separated by electrophoresis through a 1.2% agarose gel, blotted to a nylon membrane (Schleicher & Schuell), and then probed with an rbB-I ^{32}P-radiolabeled oligonucleotide (5′-GAGGTAGCTGAGT-TGAGAT-3′). As a positive control, 50 pg of rbB-I cDNA was amplified for 35 cycles. RNA samples that were negative for rbB-I expression subsequently tested positive for PCR amplification of rat β-actin mRNA sequences (data not shown).

RESULTS AND DISCUSSION

cDNA Cloning and Primary Structure of Rat Brain Class B Ca Channel. To isolate cDNAs encoding the full-length rbB Ca channel α-1 subunit, synthetic oligonucleotides corresponding to the previously determined rbB-10 cDNA sequence (32) were radiolabeled and used to probe a size-selected rat brain cDNA library. For this study, five overlapping cDNAs totaling >28 kb were sequenced in their entirety. The full-length sequence derived from the five overlapping rbB cDNAs consists of 9395 bp and is called rbB-I. Each region of the complete open reading frame designated rbB-I was derived from analysis of at least two independent cDNA clones (data not shown). The first in-frame ATG of rbB-I is preceded by a 5′ nontranslated region of 90 bp. The ATG is followed by an open reading frame of 7008 bp and encodes a 2336-amino acid protein (Fig. 1). The calculated molecular mass of the rbB-I protein (262 kDa) is larger than any of the previously cloned rat brain Ca channel α-1 subunits (37, 38). The termination codon of the rbB-I cDNA (TAG) is followed by a 3′ nontranslated region of 2297 bp and does not contain a poly(A) addition site or poly(A) tail.

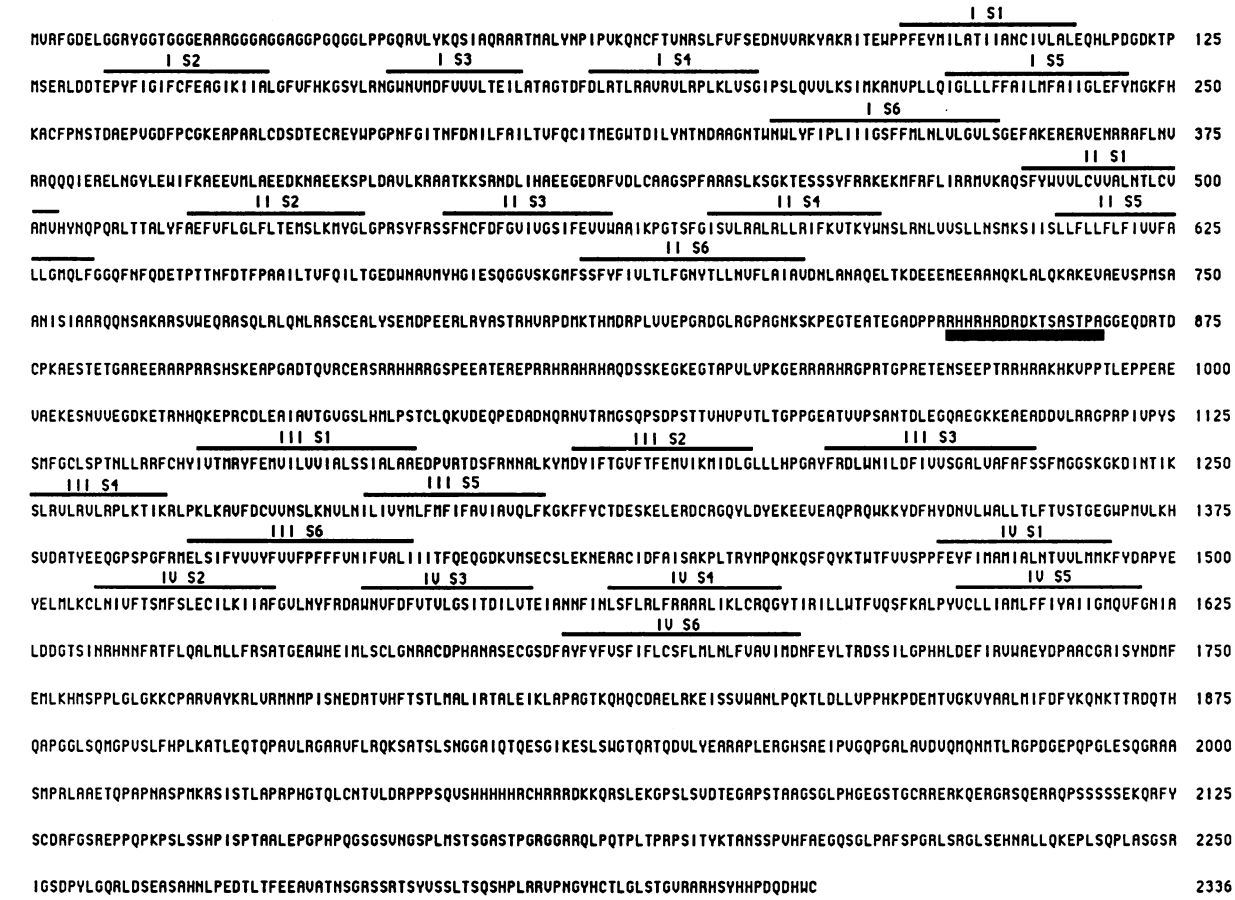

FIG. 1. Deduced amino acid sequence of the rbB-I protein. The amino acid sequence of rbB-I is shown in single-letter amino acid code. The four homology domains and the predicted transmembrane segments are aligned according to rabbit cardiac and skeletal muscle Ca channel α-1 subunits (21, 33). The thick line indicates the rbB-I peptide synthesized for antiserum generation.

The deduced amino acid sequence of rbB-I is similar in overall structure to other Ca channel α-1 subunits (21, 22, 24, 32–38). The rbB-I protein is composed of four predominantly hydrophobic homologous domains (I–IV) that are linked by hydrophilic segments of various lengths (Fig. 1). Hydrophobicity analysis reveals that each domain contains five mainly hydrophobic segments (S1, S2, S3, S5, and S6), each of which can potentially cross the membrane once. Similar to other voltage-gated ion channels, each domain of rbB-I also contains an S4 segment, in which every third amino acid is a positively charged arginine or lysine and probably represents the voltage sensor of the channel. There are 41 potential phosphorylation sites for cAMP-dependent protein kinase, cGMP-dependent protein kinase, protein kinase C, and calmodulin kinase II in the rbB-I protein (40). Thirty-seven of these consensus sites are predicted to be in cytoplasmic regions of the molecule. Three of the 13 consensus sites for N-linked glycosylation found are predicted to be on the extracellular side of the rbB-I protein.

The rbB-I protein shows only moderate identity to DHP-sensitive Ca channels (≈34% amino acid identity to cardiac, lung, and skeletal muscle L-type Ca channels). Of Ca channels cloned to date, rbB-I is most closely related to the rat brain class A (rbA) Ca channel (58% amino acid identity overall; ref. 37). Comparison of the rbA-I and rbB-I proteins shows that they are most identical in the amino terminus and in the four repeated domains (79–87% identity). Unlike cloned L-type Ca channels, both rbA-I and rbB-I contain a large hydrophilic segment (>400 residues) linking domains II and III. However, other than the region immediately flanking the carboxyl end of the domain II S6 segment, there is little identity between rbA-I and rbB-I in this region. Also of note is that both the rbA-I and rbB-I Ca channels possess a large hydrophilic carboxyl region after domain IV but show only limited amino acid identity in this region. Although the functional significance of these two major differences between the rbA-I and rbB-I Ca channels is unknown, they may reflect distinct cytoplasmic interactions involving channel modulation.

Immunoprecipitation of Brain DHP- and ω-CgTx-Binding Sites. To examine the relationship between rbB-I and the other cloned rat brain Ca channels, polyclonal antisera were generated against synthetic peptides corresponding to the amino acid sequences of the rbA-I, rbB-I, and rbC-I proteins (designated CNA 1, CNB 1, and CNC 1, respectively). The peptides comprised sequences specific for the variable domain II–domain III cytoplasmic segment of each of the cloned brain Ca channel α-1 subunits, with the rbC peptide common to both rbC-I and rbC-II splicing variants (38). Ca channels in rat forebrain membranes were labeled with either the DHP [^{3}H]PN200-110 or ^{125}I-labeled ω-CgTx (fraction GVIA), solubilized, and immunoprecipitated with the specific antiserum. As shown in Fig. 2*A*, the CNA 1 and CNB 1 antisera did not precipitate significant amounts of the PN200-110-binding sites. In contrast, the CNC 1 antiserum efficiently precipitated rat brain PN200-110-binding sites. Specificity of the CNC 1 antiserum for DHP-binding sites is indicated by the effective block of precipitation of PN200-110-binding sites by preincubation with purified rbC peptide but not the rbB peptide (Fig. 2*B*). Together with the high degree of similarity between the rbC-I and rbC-II proteins and the cardiac and lung DHP-sensitive Ca channels (≈95% amino acid identity), these results indicate that the rbC-I and rbC-II cDNAs encode α-1 subunits of neuronal L-type Ca channels. The results also suggest that the structurally distinct rbA-I and rbB-I proteins do not encode DHP-sensitive Ca channels.

Immunoprecipitation of forebrain membranes labeled with ^{125}I-labeled ω-CgTx shows that the CNA 1 and CNC 1 antisera precipitate only trace amounts of toxin-binding sites (Fig. 2*C*). In contrast, the CNB 1 antiserum specifically precipitates forebrain ω-CgTx-binding sites. Up to 50% of the ω-CgTx-binding sites are precipitated at the highest antiserum concentrations tested. Immunoprecipitation is effectively blocked by preincubation of the antiserum with purified rbB peptide (Fig. 2*D*), indicating the specificity of the CNB 1 antiserum for a single class of Ca channel. The forebrain membranes were labeled under conditions where only high-affinity, irreversible ω-CgTx sites are occupied. Because ω-CgTx blocks N-type Ca currents irreversibly and with high affinity (5–7), immunoprecipitation with the CNB 1 antiserum suggests that the rbB-I cDNA encodes the α-1 subunit of an ω-CgTx-sensitive N-type Ca channel. The incomplete precipitation may be due to the existence of multiple subtypes of high-affinity ω-CgTx receptor in the brain or to a low-titer antibody.

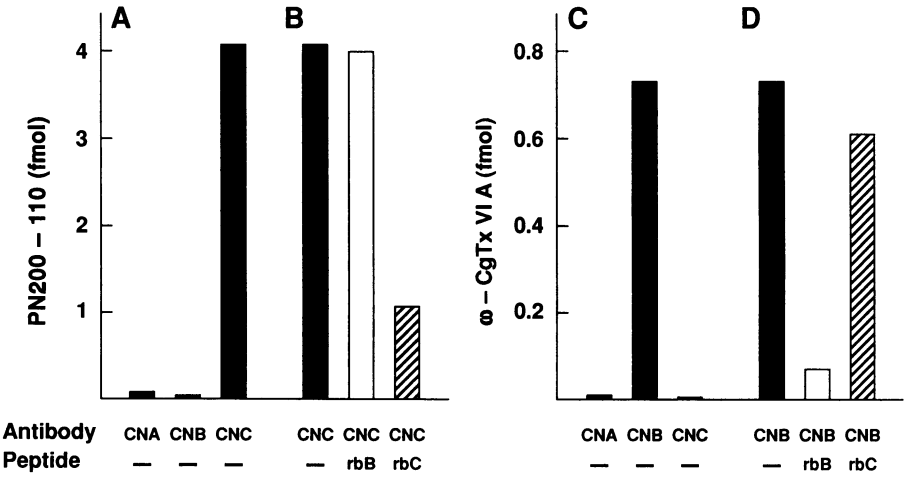

FIG. 2. Immunoprecipitation of brain Ca channels labeled with either [^{3}H]PN200-110 or ^{125}I-labeled ω-CgTx GVIA. (*A*) Immunoprecipitation of rat forebrain [^{3}H]PN200-110-binding sites by polyclonal antisera generated against the rbA (CNA), rbB (CNB), and rbC (CNC) Ca channel α-1 subunits (filled bars). (*B*) Peptide block of immunoprecipitation of PN200-110-binding sites. Antiserum CNC-1 was incubated with the rbB (open bar) or rbC (hatched bar) peptides as indicated before immunoprecipitation. (*C*) Immunoprecipitation of forebrain ^{125}I-labeled ω-CgTx-binding sites by the CNA, CNB, and CNC antisera (filled bars). (*D*) Peptide block of immunoprecipitation of ω-CgTx-binding sites. Antiserum CNB-1 was incubated with the rbB (open bar) or rbC (hatched bar) peptides as indicated before immunoprecipitation. Unspecific precipitation of receptors as determined with a nonspecific antiserum was subtracted (0.1 fmol for ^{125}I-labeled ω-CgTx and 0.33 fmol for [^{3}H]PN200-110).

The CNA 1 antiserum does not precipitate either DHP or ω-CgTx-binding sites (Fig. 2). This result is consistent with expression studies of the B-I Ca channel recently cloned from rabbit brain (24), with which rbA-I shares a high degree of amino acid sequence identity (86%; ref. 37). The rabbit B-I α-1 subunit encodes a Ca channel that is resistant to both DHPs and ω-CgTx (24) but is blocked by venom from the funnel web spider consistent with P-type Ca channels (41).

Spatial Distribution of rbB-I Expression. Autoradiographic studies of ^{125}I-labeled ω-CgTx-binding sites in the rat brain show a wide distribution of binding, with the highest densities in the cerebral cortex, hippocampus, olfactory bulb, and cerebellar cortex (42). To examine the spatial pattern of rbB-I expression, total cellular RNA was isolated from various regions of adult rat brain and probed with a 2.5-kb cDNA fragment specific for the rbB-I Ca channel. Fig. 3 shows that the probe detects a single major transcript of ≈10 kb. Significantly, the rbB-I transcript is present in all regions of the rat central nervous system, including the spinal cord. The relative levels of rbB-I expression vary slightly between regions and are quite distinct from the expression pattern of the rbA-I Ca channel, which is predominantly expressed in the cerebellum (37). The wide distribution of rbB-I transcripts agrees with autoradiographic studies of ^{125}I-labeled ω-CgTx-binding sites in rat brain and is consistent with the putative role of ω-CgTx-sensitive Ca channels in neurotransmitter release.

The distribution of rbB-I expression in other rat tissues was examined by using the highly sensitive PCR assay. First-strand cDNA was synthesized from total RNAs by using a downstream primer specific for the 3′ nontranslated region of rbB-I. Subsequently, the cDNA was amplified by using the downstream primer and a second rbB-I-specific upstream primer. Examination of the PCR products by agarose gel electrophoresis showed that a single 367-bp fragment resulted from brain RNA (data not shown). Hybridization of a radiolabeled oligonucleotide specific for the 3′ nontranslated region of rbB-I confirmed that rbB-I transcripts are detected only in brain and not in other rat tissues (Fig. 4*A*). Similar results were obtained by using a second set of rbB-I primers specific for the domain II–III segment of rbB-I (data not shown). The rbB-I tissue distribution is distinct from that of rbA-I, which is expressed in rat brain, heart, and pituitary (37). Similarly, the rbB-I expression pattern is distinct from the rbC-I and rbC-II patterns, which are expressed in all tissues examined (38). Thus, rbB-I is the only cloned Ca channel demonstrated to be specifically localized to the nervous system.

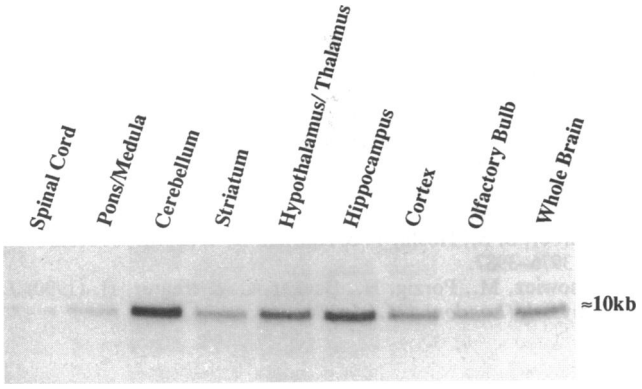

FIG. 3. Northern blot analysis of rbB-I expression in the rat central nervous system. Autoradiograph of blot hybridization of a 2.5-kb cDNA probe specific for the 3′ noncoding region of rbB-I to 30 μg of various rat brain total RNAs. Size of the rbB-I transcript was determined by using RNA standards (BRL). Autoradiography was for 7 days with an intensifying screen.

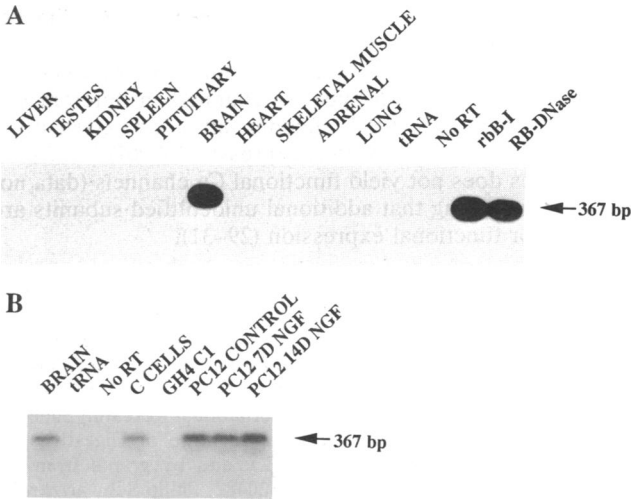

FIG. 4. Distribution of rbB-I expression in rat tissues and cell lines. (*A*) Autoradiograph of hybridization of a ^{32}P-labeled rbB-I-specific oligonucleotide to PCR products from various rat tissue RNAs. (*B*) Autoradiograph of hybridization of the rbB-I probe to PCR products from RNA isolated from rat cell lines. The PC-12 samples include RNA isolated from cells grown in the absence of nerve growth factor (NGF) (PC-12-uninduced) or 7 and 14 days after addition of NGF at 50 ng/ml to the culture medium. For *A* and *B*, negative controls include a sample in which yeast tRNA was amplified and a brain RNA sample where no reverse transcriptase was added (no RT). Positive controls include amplification of the rbB-I cDNA and a rat brain RNA sample pretreated by digestion with DNase I (RB-DNase). In both *A* and *B* the amplified PCR product is 367 bp. Autoradiography was for 6 hr with an intensifying screen.

Expression of rbB-I in Rat Cell Lines. To probe the relationship between the rbB-I protein and N-type Ca channels further, we examined the expression of rbB-I in several rat cell lines that have been shown to possess various voltage-gated Ca channels. Whole-cell patch-clamp studies of prolactin and growth hormone secreting GH_4C_1 pituitary cells have identified only T- and L-type Ca channels in these cells (43). PCR amplification of RNA from GH_4C_1 cells did not produce an rbB-I product, indicating that the rbB-I Ca channel is not expressed in these endocrine cells (Fig. 4*B*). Calcitonin-secreting C cells originate in the neural crest, but during embryogenesis these cells migrate to the thyroid gland, where they function as endocrine cells (44). Rat pheochromocytoma PC-12 cells are similar to adrenal chromaffin cells, but upon exposure to NGF they cease dividing, extend neurites, and develop many characteristics of sympathetic neurons (45). Electrophysiological studies have demonstrated T-, L-, and N-type Ca channels in C cells (46) and in both undifferentiated and differentiated PC-12 cells (6, 47, 48). PCR analysis shows that the rbB-I Ca channel is expressed in C cells and undifferentiated and differentiated PC-12 cells (Fig. 4*B*). Furthermore, RNA blot analysis with an rbB-I cDNA probe indicates that C cells express an abundant rbB-I transcript similar in size to that found in rat brain (≈10 kb; data not shown). Thus, there is an exact correspondence between expression of rbB-I and the presence of N-type Ca channels in these clonal cell lines, and there is no correlation with L- or T-type Ca channels.

CONCLUSIONS

The predicted size of rbB-I is consistent with labeling experiments that identify a single 230- to 240-kDa brain polypeptide as the target of photoreactive ω-CgTx derivatives (29, 31). The presence of multiple consensus sites for phosphorylation by cAMP-dependent protein kinase and protein kinase C is consistent with the rapid phosphorylation of a 240-kDa

subunit of an immuno-purified ω-CgTx-sensitive Ca channel by both of these enzymes (31). Our immunoprecipitation data, together with the exact correspondence between expression of rbB-I and N-type Ca channels in different cell lines, suggest that rbB-I encodes an ω-CgTx-sensitive, N-type Ca channel. However, expression of rbB-I in *Xenopus* oocytes does not yield functional Ca channels (data not shown), suggesting that additional unidentified subunits are required for functional expression (29–31).

We thank Dr. Don Nelson and Mary Gilbert for their comments on the manuscript. We are grateful to Dr. Steve Vincent for help in dissection of the rat brain for RNA preparation. This work was supported by grants from the Medical Research Council of Canada, the British Columbia Health Care Research Foundation, and the Howard Hughes Medical Institute, International Research Scholars Program (to T.P.S.); by the National Institute of Digestive and Kidney Diseases (DK-40131 to J.J.E.); and by grants from the National Institutes of Health (PO1-NS20482, Philip Schwartzkroin, Principal Investigator) and the Office of Naval Research (Peter Schwindt, Principal Investigator) to W.A.C. T.V.B.S. is supported by a postdoctoral fellowship from the Medical Research Council of Canada, and J.H. is supported by a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft. T.P.S. is a recipient of a fellowship from the Alfred P. Sloan Research Foundation.

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