Biochemical Properties and Subcellular Distribution of the BI and rbA Isoforms of α1A Subunits of Brain Calcium Channels

Takashi Sakurai, Ruth E. Westenbroek, Jens Rettig, Johannes Hell, and William A. Catterall

Department of Pharmacology, Mailbox 357280, University of Washington, Seattle, Washington 98195-7280

Abstract. Biochemical properties and subcellular distribution of the class A calcium channel α1 subunits (α1A) from rat and rabbit brain were examined using site-directed anti-peptide antibodies specific for rat rbA (anti-CNA3) and for rabbit BI (anti-NBI-1 and anti-NBI-2) isoforms of α1A. In immunoblotting experiments, anti-CNA3 specifically identifies multiple α1A polypeptides with apparent molecular masses of 210, 190, and 160 kD, and anti-NBI-1 and anti-NBI-2 specifically recognize 190-kD α1A polypeptides in rat brain membrane. In rabbit brain, anti-NBI-1 or anti-NBI-2 specifically detect α1A polypeptides with apparent molecular masses of 220, 200, and 190 kD, while anti-CNA3 specifically recognizes 190-kD α1A polypeptides. These polypeptides evidently represent multiple isoforms of α1A present in both rat and rabbit brain. Anti-CNA3 specifically immunoprecipitates high-affinity receptor sites for ω-conotoxin MVIIC (Kd ~ 100 pM), whereas anti-NBI-2 immunoprecipitates two distinct affinity receptor sites for ω-conotoxin MVIIIC (Kd ~ 100 pM and ~1 μM). Coimmunoprecipitation experiments indicate that α1A subunits recognized by anti-CNA3 and anti-NBI-2 are associated with syntaxin in a stable, SDS-resistant complex and with synaptotagmin. Immunofluorescence studies reveal that calcium channels recognized by anti-NBI-2 are localized predominantly in dendrites and nerve terminals forming synapses on them, while calcium channels recognized by anti-CNA3 are localized more prominently in cell bodies and in nerve terminals. The mossy fiber terminals in hippocampus and the terminals of climbing and parallel fibers in cerebellum are differentially stained by these isoform-specific antibodies. These results indicate that both rbA and BI isoforms of α1A are expressed in rat and rabbit brain and form calcium channels having α1A subunits with distinct molecular mass, pharmacology, and subcellular localization.

Multiple types of voltage-dependent calcium channels have been distinguished on the basis of biophysical and pharmacological criteria (L, T, N, P, and Q) and may differ in their cellular distribution and functional roles (Llinas et al., 1989; Zhang et al., 1993). Voltage-dependent calcium channels are composed of five subunits (α1, α2, δ, β, and γ) (Catterall, 1988). The α1 subunit forms the ion-conducting pore and serves as a voltage sensor, while the other subunits can modulate calcium currents conducted by α1 (for review see Isom et al., 1994). Homology screening has led to the identification of five distinct classes of calcium channel α1 subunit in the rat brain, designated rbA, rbB, rbC, rbD, and rbE (Snutch et al., 1990; Snutch and Reiner, 1992; Birnbaumer et al., 1994).

Both α1A (α1 subunit of class A brain calcium channel) and α1B (α1 subunit of class B brain calcium channel) subunits have been implicated in synaptic transmission. α1B forms an N-type calcium channel that is neuro-specific and sensitive to the cone snail toxin ω-conotoxin GVIA (Dubel et al., 1992; Williams et al., 1992; Fujita et al., 1993). Class B N-type calcium channels are localized at high density in presynaptic terminals and at lower density in dendrites of central neurons (Westenbroek et al., 1992, 1995), and ω-conotoxin GVIA inhibits synaptic neurotransmission at the central and peripheral synapses (Horne and Kemp, 1991; Luebke et al., 1993; Wheeler et al., 1994; for review see Olivera et al., 1994; Dunlap et al., 1995), indicating that presynaptic N-type calcium channels are involved in synaptic exocytosis.

α1A forms high-voltage-activated calcium channels expressed abundantly in the cerebellum including the Purkinje neurons (Mori et al., 1991; Starr et al., 1991; Sather et al., 1993; Stea et al., 1994), where P-type calcium current was first recorded (Llinas et al., 1989). ω-agatoxin IVA from venom of the funnel web spider Agelenopsis aperta and ω-conotoxin MVIIC from venom of the cone snail Conus
magus inhibit both P-type and Q-type calcium currents, but ω-agatoxin IVA has higher affinity for block of P-type currents, and ω-CTx-MVIIC has higher affinity for block of Q-type currents (Ollivera et al., 1994). Calcium channels expressed in *Xenopus* oocytes from αIA cDNA have the pharmacological characteristics of Q-type (Hillyard et al., 1992; Sather et al., 1993; Stea et al., 1994; Zhang et al., 1995; Randall et al., 1995). However, similarities in physiological properties and localization of αIA subunits and P-type calcium channels suggest that specific isoforms of αIA subunits may form both P-type and Q-type calcium channels (Mori et al., 1991; Sather et al., 1993; Stea et al., 1994; Westenbroek et al., 1995; DeWaard and Campbell, 1995).

Calcium channels containing αIA subunits are widely distributed in the central nervous system and are localized in high density in the presynaptic nerve terminals of many classes of neurons and at lower density in dendrites (Ousley and Froehner, 1994; Westenbroek et al., 1993, 1995). ω-Agatoxin IVA and ω-conotoxin MVIIC inhibit excitatory glutamatergic transmission in the hippocampus (Takahashi and Momiyama, 1993; Luebke et al., 1993; Castillo et al., 1994; Wu and Saggau, 1994; Wheeler et al., 1994), inhibitory GABAergic neurotransmission in the cerebellum and spinal cord (Takahashi and Momiyama, 1993; Regehr and Mintz, 1994), and cholinergic transmission at neuromuscular junction (Uchitel et al., 1992; Bowserox et al., 1995). The inhibition of synaptic transmission by ω-agatoxin IVA occurs with distinct IC50 values in some presynaptic nerve terminals (Castillo et al., 1994). These results implicate P/Q-type calcium channels in synaptic neurotransmission at various synapses in the central and peripheral nervous system. They may be functionally more divergent and quantitatively more important than N-type channels in regulating calcium entry in the presynaptic nerve terminal (Dunlap et al., 1995).

Although calcium channels containing α1A subunits appear to have diverse functional properties and important physiological roles, little is known about the molecular basis of diversity in α1A subunits. cDNAs encoding α1A subunits have been cloned from two different species, rabbit (BII; Mori et al., 1991) and rat (rabIA; Starr et al., 1991). They share an overall amino acid sequence identity of 92%, but the intracellular loop between domains II and III (I1-I2-II) is strikingly divergent (78% identical) compared to the remainder of the protein (>98% identical in all other regions except the COOH-terminal region). This striking difference in conservation suggests that the BI and rabIA cDNAs may encode isoforms with alternatively spliced sequences in I1-I2-II. cDNAs encoding multiple isoforms of α1A have been reported for both BI and rabIA (Mori et al., 1991; Gehr et al., 1991; Stea et al., 1994), and we have identified multiple isoforms of the α1A protein with different molecular masses that appear to arise from alternative mRNA splicing (Sakurai et al., 1995). However, it has not been determined whether both the rabIA and BI isoforms are expressed in a single species. To examine whether the BI and rabIA isoforms of α1A are both expressed in a single species and may serve distinct functional roles, we detected the rabIA and BI isoforms of α1A subunits in rat and rabbit brain using specific site-directed anti-peptide antibodies and characterized their biochemical and pharmacological properties and their subcellular distribution.

### Materials and Methods

**Materials**

[1H]sidipine (PN200-110; 80 Ci/mmol) and [125I]ω-conotoxin GV1A (2,200 Ci/mmol) were purchased from Dupont-New England Nuclear (Wilmington, DE). [125I]ω-conotoxin MVIIC was a generous gift from Drs. A. Wopmann, L. Nadasdi, and P.G. Miljanich of Neurex Corp. (Menlo Park, CA). Tricine precast gel was obtained from Novex (San Diego, CA), enhanced chemiluminescence (ECL) detection kit for immunoblotting from Amersham Corp. (Arlington Heights, IL), digitonin from Gallard-Schlesinger Chemical Mfg. Corp. (Carle Place, NY), and protein A-Sepharose (PAS) and heparin-agarose from Sigma Chemical Co. (St. Louis, MO). Expression vector pGEX-4T and glutathione-Sepharose 4B were obtained from Pharmacia LKB Biotechnology (Piscataway, NJ). All other reagents were of standard biochemical quality from commercial sources. Monoclonal anti-synaptotagmin antibodies, designated mAb10H5 and mAb1D12, respectively, were generous gifts from Dr. Masami Takahashi, Mitsubishi-Kasei Life Science Institute, Tokyo, Japan (Yoshida et al., 1992; L6v~que et al., 1992).

**Production and Purification of Peptides and Anti-peptide Antibodies**

The peptide CNA3 ([KY]SEPQREHAPPREHV) corresponds to residues 882-896 and the peptide CNA1 ([KY]PSSPERAPGREGPYGRE) corresponds to residues 886-891 of rabIA isolated from rat brain (Starr et al., 1991; Sakurai et al., 1995; Westenbroek et al., 1995). The peptide NBI-1 ([KY]TVDORLGQQRAEDFLRK) and the peptide NBI-2 ([KY]QAREGGLEPPGF) correspond to the residues 845-861, and 904-918 of BI isolated from rabbit brain, respectively (Mori et al., 1991). These four peptide sequences are located in a highly variable segment in the intracellular loop between domains II and III of the α1A subunit of brain calcium channels. The NH2-terminal lysine and tyrosine are not part of the channel sequence and were added for cross-linking and labeling purposes. The CNA1, CNA3, NBI-1, and NBI-2 peptides were synthesized by the solid phase method (Merrifield, 1963), and then purified by reversed-phase high pressure liquid chromatography on a Vydac 281TP10 column (Hesperia, CA). The identities of the purified peptides were confirmed by amino acid analysis. The purified peptides were coupled through amino groups with glutaraldehyde to BSA, diazoyzed against PBS, and emulsified in an equal volume of Freund's complete (initial injection) or incomplete adjuvant. The coupled peptides were injected into multiple subcutaneous sites on New Zealand White rabbits at 3-wk intervals. Antiserum was collected after the second injection and tested by enzyme-linked immunosor- bent assay using microtiter plates with wells coated with 0.5 μg of peptide (Posnett et al., 1988). Antibodies were purified by affinity chromatography on the corresponding peptides coupled to CNBr-activated Sepharose. 2 ml of the antiserum were bound to the column at 4°C overnight and washed with TBS. The bound IgG was eluted with 5.0 M MgCl2. The affinity-purified antibodies were then dialyzed against TBS using a Centriprep 30 (Amicon Corp., Danvers, MA).

**Preparation of Fusion Proteins and Anti-fusion Protein Antibodies**

Glutathione-S-transferase (GST) fusion proteins of α1A (CN5 and CAN6) were generated using cDNAs encoding the rabIA isoform (Starr et al., 1991) or the human BI (b96) isoform derived from a human small cell carcinoma cell line (Rettig et al., 1993), respectively, as a template and synthetic oligonucleotides with overlapping restriction sites as primers in a PCR. Sequences corresponding to residues 842-981 of rat rabIA and 569-712 of the human BI clone were amplified by PCR and cloned into the pGEX-4T expression vector (Pharmacia LKB Biotechnology) to obtain in-frame recombinant proteins containing GST. All constructs were verified by DNA sequencing and transformed into a protease-deficient strain BL26 of *Escherichia coli* (Novagen Inc., Madison, WI). Fusion protein expression was performed following the basic protocol of Sambrook and Johnson.
(1988). In brief, fresh overnight cultures were diluted 1:10 in Luria broth containing ampicillin (100 μg/ml), incubated for 90 min at 37°C with shaking, and induced by addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside. After 2-4 h of growth, bacterial cultures were pelleted by centrifugation at 5,000 g for 10 min at 4°C and resuspended in PBS containing protease inhibitors (4 μg/ml pepstatin, 4 μg/ml aprotonin, 4 μg/ml leupeptin, 0.4 mM PMSF). The bacteria were then lysed by mild sonication, solubilized by adding Triton X-100 to a final concentration of 1%, and incubated for 30 min on ice. The suspension was centrifuged at 15,000 rpm for 15 min, and the supernatant was stored in aliquots at −20°C.

For the production of anti-fusion protein antibodies (anti-CNA5 and anti-CNA6), CNA5 and CNA6 were emulsified in Freund's complete or incomplete adjuvant and injected in New Zealand White rabbits. Antibodies were affinity purified from antisera as described above for the production of anti-peptide antibodies.

**Immunoblotting of GST Fusion Proteins and Calcium Channels in Rat and Rabbit Brain**

For immunoblotting of CNA5 and CNA6 fusion proteins, 5-10 μg of each fusion protein was treated in SDS sample buffer (200 mM Tris-HCl, pH 6.8, 10 mM dithiothreitol, 4 mM urea, 8% SDS, 10% glycerol), separated by SDS-PAGE in 10% acrylamide gels, and transferred onto a nitrocellulose membrane (0.2 μm) in a buffer containing 12.5 mM Tris, pH 8.3, 96 mM glycine, 0.1% SDS, 15% (vol/vol) methanol. Unbound sites on the nitrocellulose were blocked for 2 h at room temperature with TBS containing 10% skim milk powder and incubated with affinity-purified anti-CNA1, anti-CNA3, anti-NBI-1, or anti-NBI-2 antibodies (10-30 μg/ml) in TBS for 2 h at room temperature. After five 5-min washes, blots were incubated for 1 h with HRP-protein A diluted 1:2,000 in TBS. After another 10-min washes, the blots were developed with the ECL reagent. For peptide block, the corresponding peptides at 2 μM were added to the affinity-purified antibodies and incubated overnight on ice before incubation with samples.

Brain membranes were usually prepared from 15-20-old Sprague-Dawley rats, or from 3-5-mo-old New Zealand White rabbits. Brain calcium channels were solubilized with 12% digitonin and partially purified by the chromatography on WGA-Sepharose as described previously (Westenbroek et al., 1992, 1995).

For the immunoblotting of rat and rabbit brain membranes, calcium channels in the WGA-column fractions containing 0.1 mg of total protein were concentrated by incubation with 40 μl of heparin-agarose for 4 h on ice (Sakamoto and Campbell, 1991). The resin was washed three times with 10 mM Tris-HCl, pH 7.4, 0.1% digitonin, and then with 10 mM Tris-HCl, pH 7.4. Calcium channels were extracted for 30 min at 50-60°C with 30 μl of SDS sample buffer. After analysis by SDS-PAGE, proteins were transferred onto a nitrocellulose membrane and blocked for 1 h at room temperature with TBS containing 0.1% BSA. Nitrocellulose membranes were incubated with affinity-purified anti-CNA3, anti-NBI-1, or anti-NBI-2 antibodies (10-30 μg/ml) in TBS for 2 h at room temperature. After five 5-min washes, blots were incubated for 1 h with HRP-protein A diluted 1:2,000 in TBS. After another 10-min washes, the blots were developed with the ECL reagent. For peptide block, the corresponding peptides at 2 μM were added to the affinity-purified antibodies and incubated overnight on ice before incubation with the samples.

**Radioactive Ligand Binding Experiments**

For [3H]PN-200-110 (isradipine) binding studies, 40 μl of S1 fraction was preabsorbed for 30 min on ice with 150 μl of 15% WGA-Sepharose CL-4B and incubated for 1.5 h on ice with 10 μg of PAS, and incubated for 4 h on ice in a Tilting mixer. Samples were washed quickly three times in 10 mM Tris-HCl, pH 7.4, 75 mM NaCl, 0.1% digitonin, 0.2% BSA. [3H]PN-200-110 binding in the pellet was measured using 100 μl of the labeled S3 fraction in the filter-binding assay described above for [3H]PN-200-110. [3H]PN-200-110 binding was measured by incubation of 400 μl samples containing 140 μl of WGA extract, 10 mM Tris-HCl, pH 7.4, and 0.2% BSA with 0.1 μCi of labeled toxin (1,300 Ci/mmol) at a concentration of 0.15 nM on ice for 30 min. This was added to 15 μg of affinity-purified anti-CNA3, anti-NBI-1, anti-NBI-2, anti-CNCl, or control rabbit IgG, coupled to 2 mg of PAS, and incubated for 4 h on ice in a Tilting mixer. Samples were washed quickly three times in 10 mM Tris-HCl, pH 7.4, 75 mM NaCl, 0.1% digitonin, 0.2% BSA. [3H]PN-200-110 binding in the pellet was counted in a gamma counter. Total [3H]PN-200-110 binding was determined using 100 μl of the labeled S3 fraction in the filtering assay described above for [3H]PN-200-110.

**Commmunoprecipitation Studies of α1A with Synaptic Proteins**

For the communoprecipitation experiments of α1A with syntaxin and synaptotagmin, a rat brain membrane fraction was prepared according to the procedures described by Lévéque et al. (1994). Briefly, five rat brains were dissected and homogenized in a glass/Teflon homogenizer in buffer A (0.32 M sucrose, 5 mM Tris-HCl, pH 7.4, 1 mM EDTA) on ice, containing protease inhibitors (1 μg/ml pepstatin A, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 0.2 mM PMSF, 0.1 mM benzamidine, and 8 μg/ml calpain inhibitors I and II). After a 5-minute centrifugation at 800 g, the supernatant was recovered and spun at 27,000 g for 40 min. Membrane pellets were resuspended and stirred for 15 min on ice in 25 ml of buffer B (3 mM urea, 1 mM EDTA, 1 mM diethylthiocarbamate, 5 mM Tris-HCl, pH 7.4) containing protease inhibitors. 100 ml of 0.4 M potassium phosphate, 1 mM EDTA, 1 mM diethylthiocarbamate, 5 mM Tris-HCl, pH 7.4 was added to samples and incubated for 10 min on ice. The membranes were recovered by centrifugation and solubilized in 50 ml of 1% CHAPS in 100 mM NaCl. Unsolubilized material was removed by the centrifugation (42,000 rpm, 40 min, Ti 45 rotor), and the supernatant (S3) was stored at −80°C.

0.3 ml of S3 fraction was preabsorbed for 30 min on ice with 150 μl of Sepharose Cl-4B and incubated for 1.5 h on ice with 100 μg of control IgG, followed by incubation for 1.5 h on ice with 10 μg of PAS to remove complexes of control IgG with nongeneric binding proteins. After a 1-min centrifugation on a table-top centrifuge, the supernatants were collected and incubated for 3 h on ice with either anti-CNA3 (40 μg), anti-NBI-2 (40 μg), anti-CNCl2 (20 μg), anti-SP19 (40 μg), or control antibody (40 μg). The immune complexes were recovered by the incubation on ice for 1.5 h with 4 μg of PAS, washed three times in buffer A containing 1% CHAPS, and incubated with 1% BSA, and washed once in 10 mM Tris-HCl, pH 7.4, 1% CHAPS. The pellets were extracted for 30 min at 50-60°C or boiled for 2-3 min with 20 μl of 4% SDS, 12% glycerol, and 0.45 M Tris-HCl, pH 8.45, and analyzed on a Tricine precast gel (Novex Inc, San Diego, CA). Analyzed samples were transferred onto the nitrocellulose membrane, blocked with 10% skim milk in TBST (Tris-buffer saline, pH 7.4, 0.05% Tween-20), and incubated for 1.5 h with a monoclonal anti-syntaxin antibody, mAb105 (diluted 1:60000) (Yoshida et al., 1992). The blots were washed three times in TBST, incubated for 1 h with HRP-conjugated anti-mouse IgG (1:10,000 dilution), washed, and detected with ECL reagent. For the detection of synaptotagmin, we stripped the blots and recovered by centrifugation of 100 μl of S3 fraction containing 0.2% BSA with 0.06 μCi of [125I]to-conotoxin GVIA (2,200 Ci/mmol) at a concentration of 100 nM on ice for 30 min. This was added to 15 μg of affinity-purified anti-CNCl, anti-NBI-1, anti-NBI-2, anti-CNCl, or control rabbit IgG, coupled to 2 mg of PAS, and incubated for 4 h on ice in a Tilting mixer. Samples were washed quickly three times in 10 mM Tris-HCl, pH 7.4, 75 mM NaCl, 0.1% digitonin, 0.2% BSA. [125I]to-conotoxin GVIA binding in the pellet was measured using 100 μl of the labeled S3 fraction in the filter-binding assay described above for [3H]PN-200-110. [125I]to-conotoxin GVIA binding was determined using 100 μl of the labeled S3 fraction in the filter-binding assay described above for [3H]PN-200-110.
cubated with secondary antibodies conjugated with HRP, washed, and visualized as described above.

**Immunocytochemistry**

2-mo-old Spague-Dawley rats were anesthetized with sodium pentobarbital, and then intracardially perfused with 4% paraformaldehyde in PB (0.1 M sodium phosphate, pH 7.4) containing 0.34% l-lysine and 0.05% sodium m-periodate (McLean and Nakane, 1974). After removal of the brains from the cranium, they were postfixed for 2 h, and then cryoprotected by sinking in successive solutions of 10, 20, and 30% (wt/vol) sucrose in PB at 4°C over 72 h. Sagittal and coronal sections (40 μm) were cut on a sliding microtome and processed for immunocytochemistry using the avidin–biotin complex method.

Free-floating sections were processed for immunofluorescence by rinsing the tissue in 0.1 M Tris buffer for 5 min, and then rinsing in 0.1 M TBS. To block biotinylated compounds, the tissue was rinsed in a 1% avidin solution in 0.1 M TBS for 30 min, rinsed in 0.1 M TBS for 20 min, put into a 1% solution of biotin in 0.1 M TBS for 30 min, and finally rinsed in 0.1 M TBS for 30 min. The tissue was then incubated in affinity-purified anti-CNA3 (diluted 1:15), anti-NBI-2 (diluted 1:30), or anti-NBI-1 (diluted 1:15) for 1 h at room temperature followed by 36 h at 4°C. All antibodies were diluted in 0.1 M TBS containing 0.1% Triton X-100 and 1% normal goat serum. The sections were rinsed for 1 h in TBS containing 0.1% Triton X-100, incubated in biotinylated goat anti-rabbit IgG (1:300 dilution) for 1 h at 37°C, rinsed in 0.1 M TBS for 15 min, rinsed in 0.1 M Tris buffer for 30 min, mounted onto gelatin-subbed slides, and then coverslipped using Vectashield (Vector Labs, Inc., Burlingame, CA). The immunofluorescence staining was viewed using a confocal microscope (600 MRC; Bio Rad Laboratories, Hercules, CA).

To determine the level of nonspecific staining, some of the control sections were incubated without primary antibody. Other control sections were incubated in primary antibody that had been preincubated for 6-8 h with the corresponding peptide (20 μM) or an unrelated peptide before incubation of the antibody with the tissue. The staining procedure was then carried out as described above. In all of the control sections, the staining patterns reported in this study were abolished in sections treated without primary antibody and when the primary antibody was blocked by the corresponding peptide. The pattern of immunoreactivity remained unaltered when an unrelated peptide was incubated with the primary antibody.

**Results**

**Immunohistochemical Characterization of Site-directed Anti-peptide Antibodies**

Calcium channel α1 subunits are composed of four predominantly hydrophobic homologous domains (I–IV) that are linked by intracellular hydrophilic loops of various length. The homologous domains exhibit a high degree of sequence conservation between subtypes, but the large intracellular loops connecting domains II and III of the neuronal calcium channel α1 subunits are highly variable and characteristic for each class of channels. The BI and rbA isoforms of α1s are different enough in amino acid sequence in the intracellular loop between domains II and III that we were able to select sequences specific for rabbit BI (NBI-1 and NBI-2) and for rat rbA (CNA3) for the production of site-directed anti-peptide antibodies (Fig. 1). The peptide sequence NBI-1 is present in BI, but it is deleted at the equivalent position of rbA. NBI-2 and CNA3 sequences are present at the corresponding positions in BI and rbA, but 11 amino acids are different out of 15 residues. The CNA1 sequence is immediately on the NH2-terminal side of CNA3 in rbA (Sakurai et al., 1995; Westenbroek et al., 1995) and is 60% identical in the equivalent segment of BI (Fig. 1). Thus, antibodies against CNA-1 may be expected to recognize both isoforms. The corresponding peptides were synthesized, coupled to BSA as a carrier, and used as an antigen for polyclonal antibody production as described in Materials and Methods. In addition, two GST fusion proteins (CNA5 and CNA6) were generated using cDNAs encoding rbA and human BI (hBI), respectively, according to the procedures described in Materials and Methods. CNA5 and CNA6 include the first half of the intracellular loop connecting domains II and III of rbA and BI, respectively, containing the CNA-1, CNA-3, NBI-1, and NBI-2 sequences. In this region, hBI is 90% identical to rabbit BI, while only 33% identical to rbA, and hBI contains identical NBI-1 and NBI-2 sequences as shown in Fig. 1.

The specificity of the polyclonal affinity-purified anti-peptide antibodies (anti-NBI-1, anti-NBI-2, anti-CNA1, and anti-CNA3) for BI and rbA was tested by immunoblotting using CNA5 and CNA6 fusion proteins as antigens (Fig. 2). When CNA5, a GST–rbA fusion protein with a predicted molecular mass of 45 kD, was loaded onto the SDS–polyacrylamide gel, affinity-purified anti-CNA1 and anti-CNA3 antibodies recognized the same immunoreactive band with an apparent molecular mass of 45 kD (Fig. 2 A, lanes 1 and 2). This immunoreactive band was specifically recognized by anti-CNA3, since the preincubation of 2 μM CNA3 peptide completely blocked the interaction of anti-CNA3 with the 45-kd immunoreactive polypeptide, but 2 μM NBI-2 peptide did not affect the immunoreactivity of anti-CNA3 (Fig. 2 A, lanes 3 and 4). In contrast, affinity-purified anti-NBI-1 and anti-NBI-2 antibodies did not reveal any immunoreactive polypeptides in the parallel experiments (Fig. 2 A, lanes 5 and 6). When CNA6, a GST–BI fusion protein with a calculated molecular mass of 45 kD, was analyzed by SDS-PAGE, affinity-purified anti-NBI-1 and anti-NBI-2 antibodies detected the immunoreactive band with an apparent molecular mass of 45 kD and smaller proteins that may be degradation products (Fig. 2 B, lanes 3 and 4). The 45-kD immunoreactive polypeptide was specifically recognized by anti-NBI-2 because preincubation with 2 μM NBI-2 completely blocked the staining of these immunoreactive bands (Fig. 2 B, lane 3). Anti-CNA1 also detected the 45-kD immunoreactive polypeptides, indicating that anti-CNA1 cross-reacts with the corresponding segment of BI (Fig. 2 B, lane 1). In contrast, affinity-purified anti-CNA3 did not recognize any immunoreactive polypeptide (Fig. 2 B, lane 2). These results indicate that affinity-purified anti-CNA3 specifically recognizes rbA, but not BI isoforms of α1s, while affinity-purified anti-NBI-1 and anti-NBI-2 are specific antibodies recognizing BI, but not rbA isoforms of α1s. Anti-CNA1 is not a specific antibody, and therefore it recognizes both BI and rbA in the immunoblot preparation. Based on these immunohistochemical properties, we used affinity-purified anti-NBI-1, anti-NBI-2, and anti-CNA3 antibodies to identify BI and rbA isoforms of α1s in rat and rabbit brain.

**Identification of α1s Polypeptides by Anti-CNA3, Anti-NBI-1, and Anti-NBI-2 in Rat and Rabbit Brain Membranes**

To identify α1s polypeptides by anti-peptide antibodies specific for BI and rbA, glycoprotein fractions from rat
Figure 1. Design of site-directed anti-peptide antibodies against α1A, and GST fusion proteins. Alignment of amino acid sequence (in single letter code) in the first half segment of the intracellular loop between domain II and III of α1A subunits in rat (top) and in human (bottom) (rbA-I; Starr et al., 1991), rabbit (middle) (BI; Mori et al., 1991), and human (bottom) (h96; Rettig et al., 1996). Amino acid identity is indicated by a dot, and gaps are indicated by spaces. The putative transmembrane segment S6 in domain II is enclosed with solid lines. Peptides (NBI-1, NBI-2, CNA1, and CNA3) chosen to produce site-directed anti-peptide antibodies are illustrated by lines above the protein sequence. Note that amino acid sequences in NBI-1, NBI-2, and CNA1 are completely conserved in rabbit BI and human h96. The arrowheads indicate the first and last residues of the CNA5 (solid arrowheads) and CNA6 (open arrowheads) fusion proteins that were generated using the rbA (Starr et al., 1991) and h96 cDNAs (Rettig et al., 1996), respectively.

Figure 2. Specific recognition of GST-rbA and GST-BI fusion proteins by anti-peptide antibodies. (A) The CNA5 (GST-rbA) fusion protein containing the first half of the cytoplasmic loop connecting domains II and III was analyzed by 10% SDS-PAGE, transferred onto a nitrocellulose sheet, blocked, probed with affinity-purified anti-CNA1 antibody (lanes 1), anti-CNA3 antibody (lanes 2-4), anti-NBI-1 antibody (lane 5), and anti-NBI-2 antibody (lane 6), incubated with HRP-conjugated antibodies, washed, and visualized with ECL reagent, as described in Materials and Methods. Anti-CNA1 antibodies were preincubated overnight with 2 μM CNA5 peptide at the same concentration (lane 4). The migration positions of marker proteins are indicated at the left side of the gel together with their molecular masses in kD. Anti-CNA3 antibodies were preincubated with 2 μM CNA2 peptide at the same concentration (lane 4). The migration positions of marker proteins are indicated at the left side of the gel together with their molecular masses in kD. Anti-NBI-1 and anti-NBI-2 were preincubated with 2 μM NBI-2 peptide at the same concentration (lane 3). 

Brain plasma membrane glycoprotein fractions, affinity-purified anti-CNA3 antibodies revealed at least three immunoreactive α1A subunits with apparent molecular masses of 210, 190, and 160 kD (Fig. 3 A, lane 1). These immunoreactive polypeptides were specifically recognized by anti-CNA3, since immunoreactivity against these bands was absorbed by the incubation with 2 μM CNA3 peptide, but not with NBI-2 peptide at the same concentration (Fig. 2 A, lanes 2 and 3). These results agree with previous studies of the biochemical properties of the immunoreactive α1A polypeptides (Sakurai et al., 1995), indicating that the 190-kD form is the major α1A polypeptide, while a doublet with a molecular mass of 210 kD is the minor form. The 190- and 210-kD immunoreactive polypeptides are created by alternative RNA splicing rather than by posttranslational processing of NH2 or COOH termini (Sakurai et al., 1995). Multiple isoforms of α1A isolated with anti-CNA3 were visualized with affinity-purified anti-CNA3 in parallel experiments. Affinity-purified anti-CNA5 antibody specifically detected five immunoreactive polypeptides with molecular masses of 230, 210, 190, 160, and 120 kD (Fig. 3 A, lane 9). Immunoreactivity for these α1A polypeptides was completely absorbed by the preincubation with 0.2 μM CNA5 fusion protein (Fig. 3 A, lane 10). 

Affinity-purified anti-NBI-1 and anti-NBI-2 are specific antibodies to the BI isofrom of α1A that has not previously been described in rat brain. Interestingly, affinity-purified anti-NBI-1 specifically recognized an immunoreactive α1A polypeptide with an apparent molecular mass of 190 kD, and labeling was completely blocked by the preincubation of 2 μM NBI-1 peptide in the rat brain membrane glycoprotein fractions (Fig. 3 A, lanes 4 and 5). Affinity-purified anti-NBI-2 also visualized a 190-kD immunoreactive α1A polypeptide (Fig. 3 A, lanes 6-8), and labeling of the 190-kD immunoreactive α1A polypeptide (Fig. 3 A, lane 5).
calcium channels were concentrated by adsorption to heparin affinity-purified antibodies in rat and rabbit brain by immunoblotting. Figure 3. Purified rat brain membrane by WGA affinity chromatography, and (A) Membrane glycoprotein fractions were isolated from solubilized rat brain membrane by WGA affinity chromatography, and calcium channels were concentrated by adsorption to heparin agarose, extracted, and analyzed by SDS-PAGE using a 5% acrylamide gel. Proteins were transferred onto a nitrocellulose membrane, blocked, incubated with anti-CNA3 (lanes 1-3), anti-NBI-1 (lanes 4 and 5), anti-NBI-2 (lanes 6-8), or anti-CNA5 (lanes 9 and 10), incubated with HRP–protein A, washed, and detected with ECL reagent, as described in Materials and Methods. For peptide block, anti-CNA3 antibodies were preincubated overnight on ice with 2 μM CNA3 peptide (lane 2) or with 2 μM NBI-1 peptide (lane 3). Anti-NBI-1 was preincubated with 2 μM NBI-1 peptide (lane 4) and anti-NBI-2 antibodies were preincubated with 2 μM NBI-2 peptide (lane 5) or with 2 μM CNA3 peptide (lane 6). Anti-CNA5 was preincubated with 2 μM CNA5 fusion protein peptide (lane 7). Multiple immunoreactive O1A polypeptides in rabbit brain were also specifically recognized by anti-CNA6 in Fig. 3 A. The 210-kD phosphorlated α1A polypeptides isolated by anti-NBI-1 and -NBI-2 were not detected by immunoblotting, probably because of their low quantity in situ.

If rB1 and rB are isoforms of α1A in the rat, it seemed likely that an isoform of α1A with a sequence related to LII-III of rat rB1, referred to as rB isoform of α1A, is also present in rabbit brain. Therefore, rabbit brain glycoprotein fractions were prepared and analyzed by SDS-PAGE as described above, and rabbit α1A polypeptides were detected by immunoblotting with site-directed anti-peptide antibodies (Fig. 3 B). Affinity-purified anti-NBI-1 antibodies visualized multiple immunoreactive α1A polypeptides with apparent molecular masses of 220, 200, and 190 kD (Fig. 3 B, lane 4). Immunoactivity against all three bands was absorbed by the preincubation of 2 μM NBI-1 peptide (Fig. 3 B, lane 5). Multiple immunoreactive α1A polypeptides in rabbit brain were also specifically recognized by affinity-purified anti-CNA6 antibody, which was raised against GST–B1 fusion protein (Fig. 3 B, lanes 9 and 10). The largest immunoreactive polypeptide with an apparent molecular mass of 220 kD was often observed as a doublet band in a high resolution autoradiogram, but the 200- and 190-kD immunoreactive α1A polypeptides were the major size forms in rabbit brain membranes. Affinity-purified anti-NBI-2 specifically recognized the 190-kD immunoreactive polypeptide of α1A, since this band was blocked by the preincubation with 2 μM NBI-2 peptide but was not affected by preincubation with CNA3 peptide at the same concentration (Fig. 3 B, lanes 6-8). Affinity-purified anti-CNA3, an rB1-specific site-directed anti-peptide antibody, detected a major immunoreactive polypeptide with an apparent molecular mass of 190 kD and a minor polypeptide of 230 kD in rabbit brain (Fig. 3 B, lane 7). Preincubation with 2 μM CNA3 peptide, but not with NBI-2 peptide at the same concentration, completely blocked the interaction of anti-CNA3 with the 190- and 230-kD immunoreactive α1A polypeptides (Fig. 3 B, lanes 2 and 3). These results demonstrate that the rB1 isoform of α1A is present in the rabbit brain, in addition to the BI isoform, although cDNA encoding rB1 was not identified in a rabbit brain cDNA library (Mori et al., 1991). Evidently, rB1 and BI are isoforms of α1A present in both rat and rabbit brain.
Immunoprecipitation of High Affinity Receptor Sites for Dihydropyridines, \(\alpha\)-conotoxin GVIA, or \(\omega\)-conotoxin MVIIIC

To determine the pharmacological properties of \(\alpha_{1A}\) poly-peptides recognized by anti-CNA3, anti-NBI-1, and anti-NBI-2, we labeled rat brain calcium channels with either \([^3H]PN200-110\), \([^{125}I\]\(\omega\)-conotoxin GVIA, or \([^{125}I\]\(\omega\)-conotoxin MVIIIC, and immunoprecipitated labeled rat brain calcium channels with affinity-purified anti-CNA3, anti-NBI-1, or anti-NBI-2 antibodies. \([^{3H}]PN200-110\) is a dihydropyridine that specifically binds to L-type calcium channels containing \(\alpha_{1C}\) and \(\alpha_{1D}\) (Dube\'l et al., 1992). At a concentration of \([^{3H}]PN200-110\) chosen to saturate all binding sites in a rat brain homogenate, affinity-purified anti-CNC1, an \(\alpha_{1C}\)-specific site-directed anti-peptide antibody, immunoprecipitated 40% of total \([^{3H}]PN200-110\) receptors, while anti-CNA3, anti-NBI-1, and anti-NBI-2 antibodies immunoprecipitated <1%, a value similar to that obtained with control rabbit IgG (Fig. 4 A). The binding of \([^{125}I\]\(\omega\)-conotoxin GVIA, a selective conotoxin blocker of N-type calcium channels containing \(\alpha_{1B}\), was tested similarly. Affinity-purified anti-CNB2, an \(\alpha_{1B}\)-specific site-directed anti-peptide antibody, immunoprecipitated >80% of the total \([^{125}I\]\(\omega\)-conotoxin GVIA binding sites in rat S3 fractions. Under the same conditions, anti-CNA3, anti-NBI-1, anti-NBI-2, and control rabbit IgG immunoprecipitated <4% of the total \([^{125}I\]\(\omega\)-conotoxin GVIA receptors (Fig. 4 B). \(\omega\)-conotoxin MVIIIC blocks Q-type calcium channels containing \(\alpha_{1A}\) at a low concentration when expressed in Xenopus oocytes (Hillyard et al., 1992; Sather et al., 1993; Stea et al., 1994). A saturating concentration of \([^{125}I\]\(\omega\)-conotoxin MVIIIC was added to solubilized rat brain membrane fractions enriched by affinity-chromatography on WGA-Sepharose. Affinity-purified anti-CNA3, anti-NBI-1, and anti-NBI-2 antibodies effectively immunoprecipitated \([^{125}I\]\(\omega\)-conotoxin MVIIIC receptors (Fig. 4 C), while control rabbit IgG immunoprecipitated only small amounts of \([^{125}I\]\(\omega\)-conotoxin MVIIIC binding sites. Immunoprecipitation of \([^{125}I\]\(\omega\)-conotoxin MVIIIC receptors with affinity-purified anti-CNA3 was specific, since preincubation of 20 
\(\mu\)M CNA3 peptide substantially blocked immunoprecipitation of \([^{125}I\]\(\omega\)-conotoxin MVIIIC receptors, while CNC1 and NBI-2 peptides at the same concentration did not affect the immunoprecipitation of \([^{125}I\]\(\omega\)-conotoxin MVIIIC receptors. Immunoprecipitation of \([^{125}I\]\(\omega\)-conotoxin MVIIIC receptors with affinity-purified anti-NBI-2 was significantly inhibited by the preincubation with 20 
\(\mu\)M NBI-2 peptide, but not with 20 
\(\mu\)M CNA3 peptide, confirming the specificity of immunoprecipitation. These results indicate that anti-NBI-1/anti-NBI-2 and anti-CNA3 specifically recognize distinct pools of \([^{125}I\]\(\omega\)-conotoxin MVIIIC receptors in rat brain.

Competitive displacement of specific binding of \([^{125}I]\)\(\omega\)-conotoxin MVIIIC by unlabeled \(\omega\)-conotoxin MVIIIC was tested to determine the affinity of \([^{125}I]\)\(\omega\)-conotoxin MVIIIC receptors recognized by anti-CNA3 and anti-NBI-2. A competitive displacement by unlabeled \(\omega\)-conotoxin MVIIIC was observed between 10 
\(\mu\)M and 1 
\(\mu\)M with half-maximal inhibition at \(\sim 100 \mu\text{M} \) (Fig. 4 D) in \(\omega\)-conotoxin MVIIIC receptors recognized by affinity-purified anti-CNA3, as previously reported (Sakurai et al., 1995). In contrast,
[125I]ω-conotoxin MVIIIC receptors immunoprecipitated with affinity-purified anti-NBI-2 were competitively displaced by unlabeled ω-conotoxin MVIIIC in a biphasic manner (Fig. 4 D), suggesting the possibility of more than one binding site for ω-conotoxin MVIIIC. The IC50 of the high affinity site for ω-conotoxin MVIIIC was ~100 pM, which is a similar value obtained in the ω-conotoxin MVIIIC receptors immunoprecipitated with anti-CNA3, and the IC50 of the low affinity site for ω-conotoxin MVIIIC was ~1 μM. The low affinity receptor sites for ω-conotoxin MVIIIC recognized by anti-NBI-2 were not ω-conotoxin GVIA binding sites, because anti-NBI-2 immunoprecipitated ~4% of total ω-conotoxin GVIA receptors (Fig. 4 B). Evidently, the pharmacological properties of a fraction of [125I]co-conotoxin MVIIC receptors immunoprecipitated with anti-NBI-2 are distinct from those recognized by anti-CNA3 in rat brain.

**Coimmunoprecipitation of α1A Subunits with Syntaxin and Synaptotagmin**

On the basis of immunocytochemical localization and electrophysiological findings, class A N-type calcium channels and class A P/Q-type calcium channels are implicated in fast synaptic neurotransmission (Llinás et al., 1992; Horne et al., 1991; Takahashi and Momiyama, 1993; Luebke et al., 1993; Wheeler et al., 1994; Westenbroek et al., 1992, 1993, 1995). Coimmunoprecipitation and calcium channel purification experiments have indicated that N-type channels are associated with synaptic proteins, such as syntaxin and synaptotagmin (Bennett et al., 1992; Lévêque et al., 1994, 1996), and that the N-type channel is a component of a prefusion docking complex for synaptic vesicles (for review see Jahn and Südhof, 1994). Therefore, it is likely that class A calcium channels are also associated with syntaxin and synaptotagmin. To determine if the α1A subunits recognized by anti-CNA3 and anti-NBI-2 are associated with synaptic proteins, we examined the association of α1A subunits with syntaxin and synaptotagmin by coimmunoprecipitation experiments (Fig. 5). Rat brain homogenates were washed in Tris-HCl buffer, pH 7.4, containing 3 M urea, 1 mM EDTA, and 1 mM dithiothreitol, and solubilized with 1.1% CHAPS as described in Materials and Methods. Calcium channel α1 subunits were isolated by immunoprecipitation with 40 μg of anti-CNB2 (lanes 1 and 2), anti-CNA3 (lanes 3–7), anti-NBI-2 (lanes 8 and 9), anti-SP19 (lanes 10 and 11), or control IgG (lane 12). Anti-SP19 is a site-directed anti-peptide antibody against type II brain sodium channel (West et al., 1991). Immunoprecipitation was performed after preincubation with 20 μM CNB2 (lane 2), CNA3 (lanes 4 and 7), CNC1 (lane 7), NBI-2 (lane 9), and SP-19 (lane 11) peptides. Samples were incubated with SDS sample buffer at 50–55°C for 30 min (lanes 1–4, 10, and 11) or boiled for 2–3 min (lanes 5–9 and 12), and then analyzed by SDS-PAGE on a precast tricine gel, blotted, and probed with mAbl0H5, an mAb against syntaxin (Yoshida et al., 1992) (A), and with mAblD12, an mAb against synaptotagmin (Lévêque et al., 1992) (B). The migration positions of marker proteins are indicated at the left side of the gel together with their molecular masses in kD.

The 35-kD immunoreactive band was substantially reduced but still weakly visualized in the immunoprecipitated samples after preincubation with 20 μM CNB2 and CNA3 peptide (Fig. 5 A, lanes 2 and 4), with anti-SP19, a site-directed anti-peptide antibody against brain sodium channel type IIA (Gordon et al., 1988; Fig. 5 A, lanes 10 and 11), and with nonimmune IgG (Fig. 5 A, lane 12). In contrast, the 90-kD and larger immunoreactive bands were specifically coimmunoprecipitated with anti-CNB2, anti-CNA3, and anti-NBI-2, since these immunoreactive bands were completely blocked by preincubation of 20 μM corresponding peptides (Fig. 5 A, lanes 2 and 4; data not shown for NBI-2 samples), and the 90-kD band was not visualized in the samples immunoprecipitated with anti-SP19 and with control rabbit IgG (Fig. 5 A, lane 12).

The synaptic proteins syntaxin, synaptosomal-associated protein of 25 kD (SNAP25), and synaptobrevin/vesicle-associated membrane protein (VAMP) form an SDS-resistant complex that is stable to exposure at temperature up to 60°C for 5 min and may function in synaptic vesicle docking and fusion (Hayashi et al., 1994; Champman et al., 1994). To test if the 90-kD synaptotagmin complexes detected by mAbl0H5 are stable, SDS-resistant complexes, we incu-
bated the immunoprecipitated samples for 2–3 min in boiling water instead of at 50°C and probed with mAb10H5. After boiling, mAb10H5 recognized a single immunoreactive band with a molecular mass of 35 kD in the immunoprecipitated samples with anti-CN2, anti-CN3, and anti–NB1-2 (Fig. 5 A, lanes 3–9; data not shown for CN2 samples). The signal of the 35-kD immunoreactive band was more intense than in samples that were immunoprecipitated with anti-CN3 and incubated at 50°C (Fig. 5 A, lanes 3 and 5). In boiled samples, the 35-kD immunoreactive bands were specifically coimmunoprecipitated with anti-CN3, since preincubation with 20 μM CN3 peptide completely blocked immunoprecipitation of the 35-kD band, while CN1 peptide at the same concentration did not affect coimmunoprecipitation with syntaxin (Fig. 5 A, lanes 5–7). The specificity of the 35-kD immunoreactive bands in the samples immunoprecipitated with anti–NB1-2 and anti-CN2 was tested by peptide block similarly (Fig. 5 A, lane 9; data not shown for CN2 samples). Thus, the 90-kD complex detected in samples incubated at 50°C was an SDS-resistant complex containing syntaxin and was specifically coimmunoprecipitated with the α1B and α1A subunits recognized by anti-CN2, anti-CN3, and anti–NB1-2 in rat brain.

We also tested the association of class A calcium channels with synaptotagmin, a 65-kD synaptic vesicle protein containing a transmembrane domain in NH2-terminal region and calcium binding domains (C2 repeats) in COOH terminus (Matthew et al., 1981; Jahn and Südhof, 1994). We stripped the membrane used for the detection of synaptotagmin and probed with mAb1D12 an mAb against synaptotagmin (Lévêque et al., 1992) (Fig. 5 B). In the samples immunoprecipitated with affinity-purified anti-CN2, mAb1D12 detected a single immunoreactive band with an apparent molecular mass of 65 kD, which was blocked by the preincubation with 20 μM CN2 peptide (Fig. 5 B, lanes 1 and 2). These results support previous findings that synaptotagmin is associated with class B N-type calcium channels (Lévêque et al., 1992, 1994) and that synaptotagmin is not included in the SDS-resistant complex (Hayashi et al., 1994). Synaptotagmin was specifically coimmunoprecipitated with affinity-purified anti-CN3 and anti–NB1-2 antibodies, since mAb1D12 detected much less intense bands in the immunoprecipitated samples after preincubation of the antibodies with the corresponding peptides at 20 μM (Fig. 5 B, lanes 1–9), or in samples immunoprecipitated with anti-SP19 or control rabbit IgG (Fig. 5 B, lanes 10–12). These results indicate that α1A subunits recognized by anti-CN3 and anti–NB1-2 are associated with synaptotagmin as well as with syntaxin, and therefore are implicated in synaptic vesicle docking and fusion at presynaptic terminals in the rat brain.

Differential Subcellular Localization of the rbA and BI Isoforms of α1A in the Cerebellum

α1A subunits recognized by anti-CN3 or anti–NB1-2 antibodies are differentially distributed in cell bodies, in dendrites, in punctate structures located in regions rich in synapses, and in punctate structures associated with cell bodies and dendrites. Based on previous studies showing that similar punctate clusters of α1A are presynaptic and are colocalized with presynaptic membrane protein syntaxin (Westenbroek et al., 1993, 1995), the punctate labeling pattern likely represents staining of class A calcium channels in presynaptic nerve terminals.

The most prominent staining with anti–NB1-2 and anti-CN3 was observed in the cerebellum. Both antibodies stained Ca2+ channels in the granular, Purkinje cell, and molecular layers of the cerebellum (Fig. 6). Anti–NB1-2 exhibited relatively dense staining along the length of Purkinje cell dendrites with moderate staining of the Purkinje cell body (Fig. 6 A). Punctate staining of nerve terminals was observed in both the granular and molecular layers (Fig. 6 A). This pattern of staining was similar to that observed using anti-CN3 antibodies previously (Westenbroek et al., 1995). In contrast, the most dense staining of the anti-CN3 antibody was observed in the soma of Purkinje cells, with moderate levels of staining in the dendrites of Purkinje neurons and in nerve terminals throughout the granular and molecular layers but comparatively little staining of dendrites (Fig. 6 D). No staining was observed when the primary antibody was eliminated from the procedure (not shown), and staining by each of the antibodies was blocked when they were preincubated with their corresponding peptides (Figs. 6, B and E). In contrast, the pattern of staining was unaltered when anti–NB1-2 was preincubated with the CN3 peptide (Fig. 6 C) or when anti-CN3 was preincubated with the NB1-2 peptide (Fig. 6 F). These results indicate that the anti-CN3 antibody, which is specific for the rbA isoform, and the anti–NB1-2 antibody, which is specific for the rabbit BI isoform, do not cross-react and specifically recognize α1A subunits with different subcellular distributions.

A more detailed analysis of the staining in the molecular layer of the cerebellum revealed low intensity staining with anti–NB1-2 along the surface of the Purkinje cell dendrites located in the deeper half of the molecular layer adjacent to the Purkinje cell body layer with superimposed punctate accumulations of stain representing nerve terminals (Fig. 7 A). Staining of dendritic surfaces with the NB1-2 antibody is less apparent in the distal portions of the Purkinje cell dendritic field where only a random pattern of punctate nerve terminals was observed (Fig. 7 B). Dendritic staining was less prevalent with anti-CN3 (Fig. 7 C), indicating preferential localization of the BI isoform relative to the rbA isoform in the dendrites of Purkinje neurons. However, there is relatively intense punctate staining of nerve terminals, which often appears to follow the contour of the faintly labeled dendritic branches located both proximal (Fig. 7 C) and distal (Fig. 7 D) to the Purkinje cell soma. At higher magnification, the pattern of immunoreactivity in the granule cell layer is very similar for both antibodies (Fig. 8, A and B). We observed staining of punctate structures around the cell bodies of neurons located throughout the granular layer as well as immunoreactivity in punctate structures in the surrounding glomeruli where synaptic complexes are formed among granule cells, Golgi cells, and incoming mossy fibers.

Differential Localization of rbA and BI Isoforms of α1A in the Hippocampus

In the CA1 region of the hippocampus, both antibodies
stained punctate synaptic structures. However, anti-NBI-2 staining was prominent in dendrites (Fig. 9 A), whereas staining by anti-CNA3 was more prominent in cell bodies (Fig. 9 D). Analysis at higher magnification reveals that the staining for $\alpha_{1A}$ subunits recognized by anti-NBI-2 extends along the entire length of the apical dendrites (Fig. 9, B and C). There is smooth staining along the dendritic surface with punctate labeling of nerve terminals superimposed upon the dendrites as well as punctate labeling of nerve terminals in the surrounding neuropil (Fig. 9, B and C). In contrast, anti-CNA3 antibodies labeled mainly the cell bodies of pyramidal neurons and interneurons in the CA1 region of the hippocampus (Fig. 9 D). At higher magnification, it appears that there is also punctate staining of nerve terminals in both the superficial (Fig. 9 E) and deep regions (Fig. 9 F) of the stratum radiatum.

In the CA3 region of the hippocampus, anti-NBI-2 stained predominantly the pyramidal neuron cell bodies and dendrites (Fig. 10 A). There was relatively dense, smooth staining of the proximal dendritic surface as well as some nerve terminals, which appeared as small punctate structures (Fig. 10 B). This coincides with the region of termination of the axons of the dentate granule neurons (the mossy fibers), making it difficult to determine if the mossy fiber terminals themselves are also labeled at a low level. Along the distal portions of CA3 dendrites, anti-NBI-2 labels both the dendritic surface and nerve terminals (Fig. 10 C).

In contrast with the pattern of staining with anti-NBI-2,
Figure 7. Localization of rbA and BI in the molecular layer of the cerebellum. Sagittal sections of adult rat brain were stained with anti-CNA3 or anti-NB1-2 using the immunofluorescence technique described in Materials and Methods. (A) Higher magnification view of staining with the anti-NB1-2 antibody immunoreactivity in the middle portion of the molecular layer, illustrating low intensity staining along dendrites and staining of punctate structures superimposed upon the dendritic staining and in the surrounding neuropil. (B) Section from the superficial portion of the molecular layer, demonstrating anti-NB1-2 staining in punctate structures. (C) Staining of anti-CNA3 in punctate structures located in the middle portion of the molecular layer. (D) Localization of anti-CNA3 in the superficial layer of the molecular layer in punctate structures. Bar, 25 μm.

anti-CNA3 densely stained the large nerve terminals of the mossy fibers along the proximal dendrites of CA3 pyramidal neurons, but stained the dendrites much less intensely (Fig. 10, D and E). Labeling of large nerve terminals was observed scattered throughout the cell body layer as well as along the proximal portion of the dendrites. Relatively low levels of labeling were observed on the cell bodies of CA3 neurons with the anti-CNA3 antibody (Fig. 10 D). Dense immunoreactivity was observed in punctate structures representing nerve terminals throughout the entire layer in which the apical dendrites of CA3 neurons reside (Fig. 10 F). These results indicate preferential localization of the rbA isoform in the nerve terminals of the mossy fibers making synapses on the proximal dendrites of CA3 neurons, and in the nerve terminals of other fibers making synapses on the distal dendrites of CA3 neurons.

Localization of the rbA and BI Isoforms of α1A in Dorsal Cerebral Cortex

Dense labeling of dendrites with anti-NB1-2 was observed throughout the dorsal cortex (Fig. 11, A and B). Dendritic labeling was common to all types of neurons and was associated with both large and small diameter dendrites (Fig. 11, A and B). Dense, smooth staining along the dendritic surface as well as punctate labeling of nerve terminals superimposed on the smooth dendritic staining was observed. In addition, there was labeling of the cell bodies of some cortical neurons. The most dense cell body labeling was associated with the large pyramidal neurons of layers 4/5. The cell bodies of neurons in the other layers of the dorsal cortex were sometimes lightly labeled.

The cell bodies of neurons throughout all layers of the
Figure 8. Distribution of α1A isoforms in the granule cell layer of the cerebellum. Sagittal sections of adult rat brain were incubated with anti-CNA3 or anti-NB1-2 as described in Materials and Methods. Tissue sections of the granule cell layer of the cerebellum, demonstrating the similar pattern of punctate staining around cell bodies and in regions of synaptic complexes (arrows) using either anti-NB1-2 (A) or anti-CNA3 (B) antibodies. Bar, 10 μm.

dorsal cortex were immunoreactive for anti-CNA3 (Fig. 11, C and D). At higher magnification, it is apparent that this antibody lightly labels the surface of the cell body and punctate structures in the surrounding neuropil (Fig. 11 D). Cytoplasmic staining around the nucleus by anti-CNA3 was prominent in neurons located in the dorsal cortex (Fig. 11 D). This pattern of staining was also present in the hippocampus and other regions of the brain and may represent staining of the Golgi complex where membrane proteins are processed before being inserted into the plasma membrane. In contrast with anti-NB1-2, anti-CNA3 did not stain the dendrites of cortical neurons detectably, indicating a preferential localization of the BI isoform of α1A in cortical dendrites.

Discussion

βI and rbA Isoforms of α1A Are Both Present in Rat and Rabbit Brain

Previous cDNA cloning studies defined the primary structures of the rbA and BI isoforms of α1A in rat and rabbit brain, respectively (Starr et al., 1991; Mori, 1991). It has been assumed that these two isoforms represent the same gene product in two distinct species. We were led to examine this issue more closely by comparison of the amino acid sequences of each segment of these two isoforms. This comparison reveals that the intracellular loop between domains II and III (LII-III) is strikingly divergent (only 78% identical) compared with the remainder of the protein (>98% identical in all other regions except the COOH-terminal region). In these experiments, we have used anti-peptide antibodies to provide direct evidence that both the BI isoform of α1A containing the NB1-1 and/or NB1-2 sequences and the rbA isoform of α1A containing the CNA3 sequence are present in both rat and rabbit brain by immunoblotting, immunoprecipitation, and immunocytochemistry. Sequence NB1-1 has been identified only in the rabbit BI isoform, while the NB1-2 and CNA3 sequences are present at corresponding positions in cDNAs encoding BI and rbA, respectively (Fig. 1). Our results and the comparison of sequence identity in different regions of the BI and rbA sequences suggest that alternative mRNA splicing may give rise to the difference in amino acid sequence in LII-III between these two distinct cDNAs (BI and rbA) encoding class A calcium channel α1 subunits. Consistent with alternative splicing in this region, an insertion/deletion of 349 residues (772-1,120) in the intracellular loop between domains II and III was identified in the initial cloning of the BI isoform (Mori et al., 1991). Anti-CNA3 and anti-NB1-2 would not be able to recognize isoforms of α1A with this segment deleted. Therefore, there may be additional isoforms of α1A that are not detected in our experiments. To identify all of the alternative splicing sites in the intracellular loop between domains II and III of α1A, an analysis of intron/exon organization of the α1A gene is required.

The amino acid sequence similarity between the BI and rbA isoforms is also relatively low in the COOH-terminal region (85%). For rabbit BI, two different length mRNAs have been isolated, differing in the COOH-terminal region (BI-1 and BI-2) (Mori et al., 1991). An additional substitution was identified in the COOH terminus of BI (sequence a and sequence b containing 28 amino acids, residues 1,857-1,884), resulting in two more potential combinations that may be represented in mRNAs. In rat rbA, four distinct transcripts were detected by Northern blot analysis (Starr et al., 1991) and two isoforms (rbA-I and rbA-II) that are apparently products of alternative RNA splicing (Soong et al., 1994) were identified. Sequence b is also present in the COOH terminus of rbA, at the equivalent position as in BI. Thus, it is likely that there is also extensive alternative splicing of the COOH terminus of α1A and that multiple combinations of alternative exons in LII-III and the COOH terminus may result in further diversity of the isoforms of α1A.

Antibodies against α1A recognize a diverse array of polypeptides in rat and rabbit brain ranging in apparent molecular mass from 230 kD to 160 kD (Sakurai et al., 1995; this paper). A 190-kD polypeptide is the most prevalent size form in all cases, and there are larger size forms present in lesser amounts that are recognized by both anti-CNA3 and anti-NB1-2 in both rat and rabbit brain. Previ-
 localization of α1A isoforms in the CA1 region of the hippocampus. Sagittal sections of adult rat brain reacted with anti-CNA3 or anti-NBI-2 antibodies using the immunofluorescence technique described in Materials and Methods. (A) Tissue section stained with anti-NBI-2, demonstrating dense staining of dendritic surfaces of CA1 pyramidal neurons. (B) Higher magnification of anti-NBI-2 staining along the proximal portions of CA1 pyramidal neuron dendrites. Superimposed upon the smooth staining of dendrites is the staining of punctate structures. (C) Higher magnification of anti-NBI-2 staining along the distal portions of the apical dendrites, illustrating both smooth staining of dendritic surfaces with staining of punctate structures superimposed upon them. (D) Tissue section stained with anti-CNA3, illustrating localization of α1A in the cell bodies of CA1 pyramidal neurons. (E) A higher magnification picture of anti-CNA3 staining in the cell soma and in punctate structures in the regions of the proximal portions of the apical dendrites. (F) Anti-CNA3 staining in the stratum radiatum, illustrating the localization of α1A in punctate structures in the region where the distal dendrites of CA1 pyramidal neurons reside. Bars: (A, D, and G) 50 μm; (B, C, E, F, H, and I) 25 μm.

Pharmacologically Distinct Isoforms of α1A in Rat Brain

The rbA and BI isoforms of α1A have similar functional and pharmacological properties when expressed in Xenopus oocytes (Sather et al., 1993; Stea et al., 1994). However, immunoprecipitation of these isoforms from brain and analysis of their neurotoxin-binding properties have revealed pharmacological differences. Our pharmacological experiments using radioactive ligands demonstrated that anti-CNA3, anti-NBI-1, and anti-NBI-2 antibodies
do not immunoprecipitate either dihydropyridine or ω-conotoxin GVIA binding sites, but specifically immunoprecipitate high affinity ω-conotoxin MVIIC receptor sites. $^{125}$ ω-conotoxin MVIIC receptors immunoprecipitated with anti-CNA3 contain a high affinity site for ω-conotoxin MVIIC with $IC_{50}$ of 100 pm, whereas $^{125}$ ω-conotoxin MVIIC receptor sites immunoprecipitated with anti-NB1-2 contain two binding sites with distinct affinities for ω-conotoxin MVIIC ($IC_{50}$ of $\approx$100 pm and $\approx$1 μM). High affinity ω-conotoxin MVIIC receptor sites recognized by anti-CNA3 and anti-NB1-2 are consistent with the conclusion that $\alpha_{1A}$ forms Q-type calcium channels because ω-conotoxin MVIIC blocks $\alpha_{1A}$ currents expressed in Xenopus oocytes and Q-type currents in the cerebellar granule neurons with $IC_{50} < 150$ nM (Sather et al., 1993; Stea et al., 1994; Zhang et al., 1993; Randall and Tsien, 1995). In contrast, native P-type calcium currents recorded in cerebellar Purkinje cells are blocked by higher concentrations of ω-conotoxin MVIIC ($IC_{50}$ of 1–10 μM; Hillyard et al., 1992). Several lines of evidence suggest that both Q-type and P-type calcium channels may contain $\alpha_{1A}$ subunits. (a) P/Q-type calcium channels are dihydropyridine- and ω-conotoxin GVIA-resistant (Llinas et al., 1989; Sather et al., 1993; Stea et al., 1994). (b) $\alpha_{1A}$ transcripts are expressed in cells with P-type and Q-type calcium channels (Mori et al., 1991; Starr et al., 1991). (c) $\alpha_{1A}$ coexpressed with specific β subunits in Xenopus oo-
Figure 11. Localization of $\alpha_{1A}$ isoforms in the dorsal cerebral cortex. Sagittal sections of adult rat brain were stained with anti-CNA3 or anti-NB1-2 using the immunofluorescence technique described in Materials and Methods. (A) Tissue section stained with anti-NB1-2, illustrating the dense staining along the entire length of the dendrites of neurons located throughout the dorsal cortex. (B) Higher magnification of anti-NB1-2 staining in the dorsal cortex, demonstrating both smooth dendritic staining and staining of occasional punctate structures superimposed upon the dendritic surface. (C) Tissue section incubated with anti-CNA3, showing low levels of staining associated with the cell bodies of neurons. (D) Higher magnification of anti-CNA3 staining, demonstrating immunoreactivity associated with cell bodies and in punctate structures in the surrounding neuropil. Bars: (A, C, and E) 50 \(\mu\)m; (B, D, and F) 25 \(\mu\)m.

cytos yields barium currents with kinetics similar to P-type currents (Stea et al., 1994; DeWaard and Campbell, 1995). (d) $\alpha_{1A}$ is localized in cell bodies, dendrites, and synapses where both P-type and Q-type calcium currents are measured (Ousley and Froehner, 1994; Westenbroek et al., 1993, 1995). Thus, the low affinity sites for $\omega$-conotoxin MVIIIC recognized by anti-NB1-2 (Fig. 4) may represent native P-type calcium channels that are encoded by alternatively spliced isoforms of $\alpha_{1A}$ mRNA and have distinct pharmacology. A definitive analysis of this point would require studies of the high affinity binding of $\omega$-agatoxin IVA which distinguishes P-type and Q-type calcium channels (Randall and Tsien, 1995), but this ligand has proven difficult to use for specific binding studies with immunoprecipitated $\alpha_{1A}$ in our hands, and neither rbA nor BI yields calcium channels with high affinity for $\omega$-agatoxin IVA when expressed in Xenopus oocytes (Sather et al., 1993; Stea et al., 1994).

$\alpha_{1A}$ Subunits Interact with Proteins Involved in Synaptic Exocytosis

According to the soluble N-ethylmaleimide-sensitive (NSF) attachment protein receptor (SNARE) hypothesis of exocytosis (Sollner et al., 1993), synaptic vesicles interact with targets for fusion when NSF and a soluble NSF attachment
protein (SNAP) bind to membrane-associated SNARES that are present in the vesicle and target membranes (designated v- and t-SNAREs, respectively). In the nerve terminal, the v-SNARE is the synaptic vesicle-associated membrane protein VAMP/synaptobrevin (Baumert et al., 1989; Trimble et al., 1990) and the t-SNAREs are syntaxin (Bennett et al., 1992; Inoue et al., 1992; Yoshida et al., 1992), and SNAP25 (Oyler et al., 1989). All assemble with SNAPs and NSF into 20 S fusion particles, resulting in the tight docking of the synaptic vesicles at the active zones (Sollner et al., 1993; Calakos et al., 1994; for review see Jahn and Südhof, 1994). Neurotransmitter release is ultimately triggered by high cytoplasmic calcium concentrations of the order of 100 μM, suggesting that voltage-gated calcium channels are clustered in the active zone, docked close to synaptic vesicles (Llinäs et al., 1992). Presynaptic N-type calcium channels are associated with syntaxin (Bennett et al., 1992; Yoshida et al., 1992; Lévêque et al., 1992, 1994), synaptobrevin/VAMP (El Far et al., 1995), and SNAP25 (Lévêque et al., 1994). Interaction with syntaxin and SNAP25 is mediated by a specific sequence in LII-III (Sheng et al., 1994, 1996), and coexpression of syntaxin in Xenopus oocytes modulates the voltage dependence of inactivation of calcium channels containing αIA and αIB (Bezprozvanny et al., 1995). Synaptotagmin, a calcium-binding protein of synaptic vesicles is also coimmunoprecipitated with syntaxin (Bennett et al., 1992; Yoshida et al., 1992), and is associated with N-type calcium channels (Lévêque et al., 1992, 1994; O’Connor et al., 1993). In contrast, class C L-type calcium channels are not associated with a synaptic vesicle fusion complex (Sheng et al., 1994; El Far et al., 1995). Our results with anti-CNA3 and anti–NB1-2 show that the rbA and BI isoforms of αIA are associated with syntaxin and synaptotagmin in the rat brain, indicating that both αIA subunit isoforms are involved in synaptic exocytosis at presynaptic nerve terminals. Direct, calcium-dependent interaction of a synaptic protein interaction site in the first half of LII-III of αIB and of the BI isoform of αIA with syntaxin and SNAP25 has been observed in binding assays in vitro (Sheng et al., 1996; Rettig et al., 1996). However, the corresponding part of LII-III of rbA binds SNAP25 but not syntaxin (Rettig et al., 1996), suggesting that a structural difference in LII-III, which results from alternative RNA splicing, causes differential binding affinity for syntaxin.

An SDS-resistant complex consisting of syntaxin, SNAP25, and VAMP/synaptobrevin has been characterized in previous work (Hayashi et al., 1994; Chapmann et al., 1994; McMahon and Südhof, 1995). In our experiments, αIA subunits recognized by anti-CNA3 were specifically associated with syntaxin in an SDS-resistant complex that was stable to incubation at 50–55°C for 30 min. Synaptotagmin was associated with this complex, but its association was not SDS resistant. Our results that αIA and αIB subunits of presynaptic calcium channels are specifically associated with the SDS-resistant core complex support a central role for this complex in docking and exocytosis of synaptic vesicles.

**Differential Localization of the rbA and BI Isoforms of αIA**

In a previous study, we have shown that calcium channels containing αIA subunits recognized by the broad specificity antibody anti-CNA1 are present in low density on dendrites and some cell bodies, and are concentrated at approximately eightfold higher density in presynaptic terminals making synapses on cell bodies and on dendritic shafts and spines of many classes of neurons (Westenbrook et al., 1993, 1995). Class A calcium channels recognized by anti-CNA1 were especially prominent in the synapses of the parallel fibers of cerebellar granule cells on the dendrites of the Purkinje neurons, where their localization in presynaptic terminals was confirmed by double labeling with the synaptic membrane protein syntaxin or by microinjection of the postsynaptic marker Neurobiotin (Westenbrook et al., 1993, 1995). Anti-CNA1 staining represents the sum of the staining patterns that we observed with anti-CNA3 and anti–NB1-2 antibodies in these experiments. Similar staining of punctate structures was observed with anti-CNA3 and anti–NB1-2, indicating that both of these isoforms are present in nerve terminals. These immunocytochemical results are consistent with our immunoprecipitation experiments showing that both isoforms are associated with syntaxin and synaptotagmin. However, these isoform-specific antibodies also revealed differential distribution of the BI and rbA isoforms in both dendrites and nerve terminals.

The rbA isoform recognized by anti-CNA3 is localized predominantly in cell bodies and terminals. In contrast, the BI isoform recognized by anti–NB1-2 is located along the surface of dendrites as well as in cell bodies and nerve terminals in the cerebellum, hippocampus, and cerebral cortex. These results indicate that the BI and rbA isoforms, which are specifically recognized by these antibodies, contain different targeting information that specifies a difference in their localization in dendrites. This differential localization may also imply a functional difference for the BI and rbA isoforms of αIA. Previous studies have suggested that the dendrites of cerebellar Purkinje neurons are important sites of calcium entry (Llinäs and Sugimori, 1980; Tank et al., 1988), as are the dendrites of hippocampal pyramidal neurons (Regehr et al., 1989). Increases in intracellular calcium in cerebellar Purkinje dendrites evoked by brief pulses of high K+ are blocked by low concentrations of ω-agatoxin IVA, while ω-conotoxin GVIA and ω-conotoxin MVIIIC block only a small fraction of the intracellular calcium rise (Bindokas et al., 1993). This suggests that P-type calcium channels, which are highly sensitive to ω-agatoxin IVA, are located in Purkinje cell dendrites in high density, and, to a lesser extent, N-type and Q-type calcium channels are also present. It is possible that our anti–NB1-2 antibody recognizes the BI isoform that is localized in dendrites and forms P-type calcium channels. In contrast, the rbA isoform recognized by anti-CNA3 appears to be localized preferentially in the cell body of neurons. Localization in the cell body appears to be both cytoplasmic as well as on the cell surface. The cytoplasmic staining may represent αIA subunits that are targeted for transport to the nerve terminals. Overall, our localization studies suggest that the different isoforms of the αIA subunit have both differential and overlapping distributions along the cell bodies and dendrites of central neurons, which may position them for cell type–specific roles in integration and propagation of excitatory synaptic signals.

In many regions, such as the granule cell layer and distal...
dendritic fields of the molecular layer of the cerebellum, the pattern of staining of nerve terminals with anti-NBI-2 and anti-CNA3 appears identical. The presence of more than one isoform of α1A in a single nerve terminal may allow these channels to fine tune the voltage dependence, timing, or regulation of calcium influx, which in turn is used to control the timing and extent of secretion of fast neurotransmitters and/or neuropeptides. Distinct differences in the staining of nerve terminals by these isoform-specific antibodies were also observed. The rBa isoform recognized by anti-CNA3 is present at high concentration in the mossy fiber terminals on the proximal region of the dendrites of CA3 pyramidal neurons and in nerve terminals forming synapses on the distal dendrites of CA3 neurons (Fig. 10, D–F), while the BI isoform recognized by anti-NBI-1 is absent or present at very low levels in a small subset of these terminals (Fig. 10, A–C). These results are also consistent with the idea that the rBa isoform is a component of P-type calcium channels, since transmission at the mossy fiber synapse is highly sensitive to ω-agatoxin IVA.
